CHARACTERIZATION OF A SHRIVELED SEED TRAIT IN PEANUT (*Arachis hypogaea* L.)

BY

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Dedicated to my aunt Satyavathi Devi Kari
whose love and support have made this possible.

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Seed shriveling that severely affects seed morphology and composition in peanut is a unique type of seed mutation in peanut germplasm. This characteristic also shows a differential expression within the fully mature seed of an individual, homozygous true-breeding plant. Studies were undertaken to characterize the shriveled seed characteristic in three mutant lines, 529-A, 563-A, and 647-A in peanut (*Arachis hypogaea* L.). Studies were conducted to determine the position and time of shriveling expression in the pegging zone of shriveled genotypes. Both position of the pegs and pegging time had an effect on the expression of shriveled trait. More shriveled seed were produced in the later formed pods, present in the outer pegging zones.

The F_1, F_2, and F_3 data for crosses with shriveled lines as male parents and Sunrunner as female parent showed

the shriveled character to be inherited as a single recessive allele. No F_2 segregation was observed in the reciprocal crosses. However, these crosses segregated in the F_3 generation. No allelic differences were observed among the three shriveled lines for the shriveled trait.

Seed composition of five phenotypic classes of seed differing by degree of shriveling was studied. The most shriveled class of seed had less lipid and more sucrose compared to the normal-seeded class. Significant differences were also observed for the concentrations of total protein and seed moisture. The higher relative percentage of phospholipid to the triacylglycerol in the most shriveled seed indicated that the accumulation of triacylglycerols is severely affected in shriveled seed. Reduced quantities of certain proteins were revealed by electrophoretic gel patterns and may account for the reduction of total protein in the shriveled seed. Shriveled seed accumulated less triacylglycerol than normal seed at various developmental stages. Differences in the levels of various proteins were identified between shriveled and normal seed at various developmental stages.

Partitioning of photosynthetic assimilates to pod and seed growth, calculated using glucose cost equivalents, was lower in shriveled line 529-B, compared to the normal line Sunrunner.

CHAPTER 1
INTRODUCTION

Peanut is an important oil and food crop of the world. The cultivated peanut, *Arachis hypogaea* L. belongs to the family *Leguminosae*, tribe *Aeschnomeneae*, subtribe *Stylosanthenae*. It is a self-pollinated allotetraploid (2n=4X=40) consisting of two genomes, A and B, each likely originated from a different diploid ancestor. The A and B genomes have partial homology of the chromosomes but not enough homology for chromosome pairing, thus facilitating diploid meiotic pairing (Wynne and Halward,1989). The lack of isozyme (Grieshammer and Wynne, 1990) or RFLP (restriction fragment length polymorphism) variation (Kochert et al., 1991; Paik-Ro et al., 1992) within the tetraploid species suggests that the origin of peanut is narrowly based, possibly from a single hybrid plant that doubled its chromosome number, followed by interspecific hybridization (Paik-Ro et al., 1992). However, *A. hypogaea* L. shows extensive morphological variation within the species. It is divided into two subspecies: subspecies *hypogaea* and subspecies *fastigiata* based on morphological differences, mainly the position of the floral axes and branching pattern. The subspecies *hypogaea* is subdivided

into variety *hypogaea*; in the U.S known as runner and virginia market types; and variety *hirsuta*, the peruvian type. The subspecies *fastigiata* is divided into variety *fastigiata*, the valencia market type; and variety *vulgaris*, the spanish market type. The world peanut crop is grown on 18.3 million ha with an annual production of 19.8 million t (Fletcher et al., 1992). Over the previous decade, about 55% of the total production was crushed into oil and 35% used for other food purposes. There was a 14% increase for food use compared with 1970s. This change occurred because peanut is expanding its edible peanut and snack food markets from the USA into Europe, Canada and Japan, while still being a major vegetable oil source in the developing countries (Fletcher et al., 1992). This type of change certainly underscores a need for peanut cultivars with varied types of seed composition. Usually the peanut kernel is composed of about 50% lipids, 28% protein, 13% carbohydrates, 4% starch, 3% mineral and less than 1% reducing sugars (from Salunkhe et al., 1992). A decrease in oil content and a proportionate increase in protein or sugars may facilitate the production of low fat peanut products with better flavor and taste for specialty peanut markets. A relative increase in oil or protein may improve the per hectare yields for these components, which may be especially important in developing countries where drought may be a major constraint for obtaining higher pod yields.

Even though low oil lines have been described (Salunkhe et al., 1992), no further research on their usage has been reported. This may be due to undesirable quality or yield characteristics. On the other hand, some of the lines which showed low oil concentrations, e.g., 34% and 37% in the germplasm screening at ICRISAT, later showed normal concentrations (48.3% and 45%, respectively) when tested across twelve different environments (Dwivedi et al., 1993). After considering several reports on the oil content in peanut, Salunkhe et al. (1992) concluded that most of the commercially grown peanut cultivars contain an average of about 50% oil. These results indicate a narrow genetic variability for this trait in peanut.

Perhaps as a result of a spontaneous mutation event, a few lines identified in the University of Florida peanut breeding program showed only half to two-thirds of this average oil concentration (indicated in preliminary studies conducted at the University of Florida). The lower oil concentration is associated with a characteristic shriveling of the cotyledons when they are dried after harvest at full maturity. Further, the expression of this characteristic is also not uniform in a single true-breeding plant. As a result, a range of seed phenotypes with different levels of shriveling expression can be seen on an individual plant. Also, depending upon the genetic background, the mutation showed differences in its degree of penetrance. Therefore a

possibility exists for the development of low oil peanut lines by selecting lines expressing moderate penetrance of this characteristic. Those lines can be used in the peanut snack industry as in the case of wrinkled peas (Pisum sativum L.) and various kernel mutants of maize (Zea mays L.) which have set a trend for the use of mutants for specialty food purposes. For example, today all the canning and freezing peas are invariably the wrinkled pea types where the canned pea industry represents about 5% of the total pea industry (Gritton, 1989). Similarly, most of the sweet corn industry is using shrunken-2, brittle-2, waxy and sugary mutations for the special properties associated with these mutants (Boyer and Hannah, 1992). Besides its potential food use, the shriveled mutation in peanut can be used to study the regulation of seed development in peanut.

Regulation of Seed Lipid Biosynthesis

Studying the mutation affecting storage lipid production in seed tissues can provide some insights into the biochemical regulation of lipid biosynthesis in oil storage tissues. To date very little information is available regarding the control of lipid biosynthesis in plants (Slabas and Fawcett, 1992; Browse and Somerville, 1991). Triacylglycerols are the major reserves in oil-storing seeds. The lipids of mature peanut seed are usually composed of 95% triacylglycerols, about 2% diacylglycerols and

about 1% polar lipids (Sanders, 1980). In seed triacylglycerol synthesis, a plastid pyruvate dehydrogenase complex provides acetyl-CoA from glycolysis products in the nongreen plastids. The synthesis of malonyl-CoA by acetyl-CoA carboxylase (ACC) is the first committed step in fatty acid synthesis (Browse and Somerville, 1991). According to the models proposed for the cellular organization and compartmentalization for the seed triacylglycerol synthesis (reviewed in Slabas and Fawcett, 1992; Browse and Somerville, 1991; Roesler et al., 1994) (Fig. 1-1), the acetate entering the plastid is initially converted into acetyl-CoA and then, by acetyl-CoA carboxylase, to malonyl-CoA. Both acetyl- and malonyl-CoA are used to synthesize fatty acids up to C18 in chain length as their ACP (acyl carrier protein) derivatives. Desaturation then occurred at C18:0 by stearoyl-ACP desaturase. Fatty acids are then either incorporated into complex plastid lipids or exported to the cytoplasm, followed by a hydrolysis to their fatty acid CoAs by acyl-ACP thioesterase. Then they will be used for synthesis of phosphatidylcholine (PC) which is the major substrate for 18:1 and 18:2 desaturation. Exchange of 18:1 from CoA with the fatty acids at sn-1 and sn-2 positions of PC provides 18:2 and 18:3 for diacylglycerol (DAG) for triacylglycerol (TAG) formation. Triacylglycerols are subsequently stored in oil bodies, presumably formed from the endoplasmic reticulum.

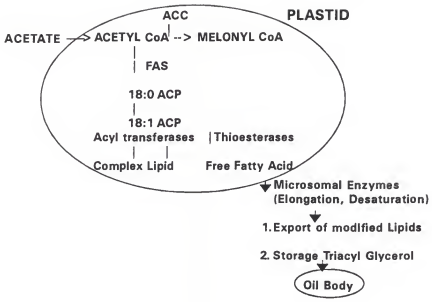

Figure 1-1. Pathway for fatty acid biosynthesis in oil seed tissues.

In animal systems it is well established that acetyl-CoA carboxylase is the rate-limiting step for fatty acid synthesis (Kim et al., 1989). In the case of rapeseed embryos, the activity of ACC increases prior to the onset of lipid synthesis and keeps increasing during lipid deposition similar to the activity of other enzymes of fatty acid synthesis. However, the activity of ACC rapidly decreases while the other enzymes of the system are still active, indicating that ACC could be a central regulator, shutting down lipid synthesis, even though an active fatty acid synthase system is present, by removing the substrate malonyl-CoA (Slabas and Fawcett, 1992). In their analysis of substrate and product pool sizes in spinach leaves, Post-Beittenmiller et al. (1991) found that fatty acid synthesis is 5-8 fold higher in light than in darkness. They observed an increase in the concentration of acetyl ACP when the plants were shifted from light to the dark. This shows that acetyl-CoA carboxylase, rather than ß-ketoacyl-ACP synthase (KAS III) is the most strongly regulated step when plants are shifted from light to dark. Nevertheless, Gibson et al. (1994) implicated the ACP (acyl carrier protein) gene expression as key to the rate of fatty acid synthesis in seed oil deposition. On the other hand, the step catalyzed by the enzyme phosphatidate phosphatase in safflower seed and the step catalyzed by diacylglycerol acyl transferase in oilseed rape are indicated to be the rate-limiting steps in

seed reserve lipid (triacylglycerol) biosynthesis (Frentzen, 1993).

Chemical Composition of Shriveled-Seed Mutants

All of the shriveled-seed mutations so far identified in crop species have shown some changes in their chemical composition. Composition studies on mature normal (RR) vs. wrinkled (rr) pea seed showed differences in the concentrations of starch, amylose, sucrose (Kooistra, 1962), lipid (Coxon and Davis, 1982), and legumin protein (Davis, 1980). Starch concentration was always greater in RR lines compared to the rr lines, whereas rr lines contain more sucrose and lipid. Legumin protein concentration was higher in RR lines compared to the rr lines. In maize, mutations at the Sh2 and Bt-2 loci give rise to kernel phenotypes which cannot be distinguished from each other. In these mutants the loss of gene function leads to inadequate starch levels and as a consequence, the seed coat or pericarp shrivels upon kernel maturation (Sullivan et al., 1991). Creech (1965) showed that the shrunken-2 allele conditions a two to three times higher concentration of sucrose and inadequate starch levels when compared to the normal types. Shrunken endosperm barley mutant *seg*8 has lower starch and higher sucrose concentration, but no significant difference in the total protein concentration (Djarot and Peterson, 1991). A shriveled Bomi-like barley mutant *shx*, described

by Schulman and Ahokas (1990), has only 25% of normal starch concentration, with only 14% of normal activity of primer-independent starch synthase enzyme. A shriveled-seed mutant in soybean (Honeycutt et al., 1989b) showed reduced level of the ß subunit of 7S storage protein. Differences were also noticed for both oil and storage protein concentration among the different shriveled types within the shrunken soybean genotype.

Reports of Turner et al. (1990) showed that higher levels of sucrose in pea embryos due to a metabolic lesion in the starch production can destabilize legumin transcripts, thereby resulting in a reduced accumulation of this storage protein in the pea mutants homozygous for r locus. The starch-deficient mutants sh2, bt2, and su2 with higher levels of sucrose in the endosperm also affect the accumulation of zein proteins (Lee and Tsai, 1984, 1985). The increased lipid content in both pea and maize embryos of the shrunken genotypes is probably because of a greater membrane area in the mutant than in the wild type embryos (Bhattacharyya et al., 1993).

One of the important studies from a series of studies on seed development in pea involving normal and wrinkled embryos (`Analysis of seed development in *Pisum sativum* L.` I through XIII) was of sucrose partitioning in those two types of seed (Edwards and Rees, 1986). Starch content per embryo for the normal types was always higher than that of

wrinkled embryos throughout their development. The embryos of round peas metabolized almost twice as much ^{14}C sucrose as did those of wrinkled peas. Round embryos incorporated most of the label into starch and greater labeling of protein was also found, but most of the label in the wrinkled types was recovered in CO_2, lipid, and other respiratory products.

The developmental analysis conducted on the accumulation patterns of the storage proteins at various developmental stages (globular, heart, early cotyledon and mature) of both shriveled and normal types in soybean (Honeycutt et al., 1989b) showed that the developmental lag time between α-subunit and ß-subunit accumulation was greater in the mutant. Also the mutant had very little accumulation of the ß subunit at maturity.

Changes in Seed Morphology as Affected by Sucrose Concentration

Changes in seed morphology to a shriveled phenotype can be caused by the decreased osmotic potential of the seed due to the accumulation of sucrose. Since the decreased osmotic potential of the seed increases water retention during seed development, shriveling of the seed results as water is lost at the end of development (Bhattacharyya et al., 1993). In the wrinkled seed mutant (rr) of pea, deficiency in the starch branching enzyme produces a lower rate of branching which would provide fewer ends of glucose chains as

substrates for starch synthase, which leads to reduced production of starch and accumulation of sucrose (Bhattacharyya et al., 1993; Smith, 1988). Several starch deficient mutants in maize are also characterized by much higher levels of sucrose in the endosperm and characteristically shrunken endosperm (Creech, 1965). The starch deficient, shrunken endosperm mutant of barley, *seg*8 also contains higher levels of sucrose (Djarot and Peterson, 1991).

Effect of Sucrose Concentration on Seed Storage Protein Accumulation

Davis (1980), using a wide range of *Pisum* genotypes, found that the mean legumin content of round-seeded lines (RR) was about twice that of wrinkled-seeded lines (rr). Turner et al. (1990) found that the levels of legumin mRNA in round-seeded isolines was much higher than in the wrinkled isolines. However the run-on transcription data suggested that there are very similar rates of transcription of legumin genes in the near isolines; therefore the differences in mRNA levels between the isolines result from increased rates of degradation of legumin mRNA in rr lines. Their *in vitro* studies with increased sucrose concentrations showed a decrease in legumin mRNA, suggesting that legumin transcripts may be selectively unstable at the more negative osmotic potential of the mutant embryo. Mutants affecting starch and zein accumulation in maize also contain higher

levels of sucrose in the endosperm (Lee and Tsai, 1984). It has been suggested that the high sucrose concentrations in the cytoplasm of these mutants alter the interaction between zein-synthesizing polyribosomes and the endoplasmic reticulum, leaving zein mRNAs more susceptible to RNase (Tsai et al., 1978). Similarly, the Class I storage protein (patatin) in potato tuber may be regulated by the sucrose levels in the cell (Rocha-Sosa et al., 1989).

Alternatively, studies on maize (Lee and Tsai,1985), pea (Beachy et al., 1985), and soybean (Holowach et al., 1984) showed that the alterations in sucrose content of the seed may affect sucrose uptake, and hence amino acid uptake, which is essential for protein synthesis in seed. Lee and Tsai (1985) reported differences between the normal and shrunken kernels in the ability to absorb radioactive amino acids. A low osmotic potential condition reduced transport of solute, but favored movement of water into kernels as indicated by increased water content. A reduced zein synthesis in the developing endosperms of all starch-forming mutants was negatively correlated with sucrose concentrations.

Inheritance of Shriveled-Seed Characteristic

Inheritance of seed morphology mutants has been extensively studied both in cereals and legumes. Maize kernel mutants bt-1, sh-2, bt-2 and mn mutations which

exhibit shrunken or collapsed phenotypes and decreased levels of endosperm starch, show single-gene inheritance (Mangelsdorf, 1926; Lowe and Nelson, 1946). Barley shrunken endosperm mutants, se1, se2, se3, se4 and se5 are also inherited as monofactorial recessives while in the se6 mutant it is a monofactorial recessive expressing xenia (Jarvi and Eslick, 1975). Shrunken endosperm mutant *seg*8 is also under the control of a single recessive allele (Ramage and Crandall, 1981). The shx mutant described by Schulman and Ahokas (1990) is also single-gene recessive. The wrinkled mutants, including r locus mutation in pea (*Pisum sativum* L.) are monogenic in inheritance (Kooistra, 1962). A shriveled-seed mutant in soybean (*Glycine max* (L.) Merr.) is monogenic recessive in inheritance (Honeycutt et al., 1989a). The monogenic recessive nature of some of these shrunken mutants is also evidenced by the recent molecular analyses of the R locus in pea which encodes a starch-debranching enzyme (Bhattacharyya et al., 1990), and Bt-1 which encodes an endosperm-bound adenylate carrier protein in maize (Sullivan et al., 1991). The Bt-2 and Sh-2 loci, each encode a subunit of endosperm ADP-glucose pyrophosphorylase in maize (Bae et al., 1990; Bhave et al., 1990). The molecular isolation of the corresponding wild type alleles of these loci led to the conclusion that the above mutations are due to a lesion in the function of a single gene.

Expression of Shriveled-Seed Trait on Plants Homozygous for Shriveled Locus

The wrinkled peas described by Kooistra (1962), the shrunken maize endosperm mutants described by Mangelsdorf (1926) and Lowe and Nelson (1946) and the shrunken barley mutants studied by Jarvi and Eslick (1975) cause all seed to be shriveled on a single homozygous plant. Differential seed shriveling was observed in certain wheat strains derived from the wheat-rye hybrids due to the interactions of rye cytoplasm with wheat nuclear background but there is no indication for nonrandom distribution of shriveling (Maan, 1984). However, the soybean shriveled-seed mutant studied by Honeycutt et al. (1989a) showed differential expression for the shriveled-seed characteristic. More shriveled seed are produced in the upper nodes of the shriveled-seed genotype compared to the lower nodes. Some shriveled seed are found on the lower nodes but none on the upper nodes of a normal-seeded line, P2180, which they used as a control in their experiments.

The three shriveled-seeded lines identified in the University of Florida peanut breeding program are not uniform in the shriveled expression within a single homozygous true-breeding plant, even though these lines were identified as true-breeding lines for several generations. The seed from the first-formed pods are usually normal but the later-formed pods show different levels of seed

shriveling. When normal seed are planted, the plants resulting from these seed again produced both normal and shriveled seed.

Peg Placement and Seed Development in Peanut

Seed development in peanut is unique. Several early workers studied the peg and fruit development in peanut (Gregory et al., 1951). After double fertilization, the meristematic cells beneath the ovary divide and develop into a downward growing, stalk-like structure called a peg. The peg is positively geotropic. Once the peg enters into the soil and places the fertilized ovule about 3 cm below the soil surface, the pod begins to enlarge as the embryo and the cotyledons begin to differentiate. The developmental morphology of peanut shows a predictable pattern of peg and fruit development on individual reproductive branches (Gupton et al., 1968). Pegs of virginia type peanuts occur first on the reproductive branch of the first pair of laterals on the main stem. Successive pegging extends outward and upward in concentric circles from the first inflorescence on the lateral, forming identifiable pegging groups depending upon when the peg penetrated the soil (Gupton et al., 1968).

One of the important earlier studies on the composition of developing peanut fruit by Schenk (1961) clearly identified weekly changes in the various developing peanut

fruit parts. Protein accumulation was very little until the fifth week after peg penetration. The oil percentage approached a maximum by the sixth week and the protein reached its maximum by the eighth week. By the ninth week the dry matter of both fruits and kernels reached their maximum value.

Pattee et al. (1974) described the characteristics of peanut fruit parts following the beginning of peg enlargement. They divided the seed development in peanut into fifteen stages of physiological maturity based upon the physiological and morphological changes in the fruit pericarp color and kernel color and texture. The dry weight of the seed increased rapidly at the initial stages of seed development, and sucrose concentration also increased throughout seed maturation. Starch accumulation was more rapid than lipid at early stages of development, but starch accumulation was subsequently slow. Lipid concentration was low initially and rate of lipid accumulation became maximum between stages 7 and 11 of kernel development. The highest amount of lipid was observed at stage 13 when the pericarp distinctly showed black splotches and the seed coat was beginning to turn brown.

Sanders (1980) reported that all lipid classes changed somewhat with maturity. Triacylglycerols increased from 85% to 96% of the lipid present. Both polar lipids and free fatty acids decreased with maturity. During the maturation

of cotyledons, palmitic, linoleic, linolenic, eicosenoic, behenic and lignoceric acids declined in concentrations while oleic acid increased (Worthington, 1969). Basha et al. (1976) reported a decrease in both free amino acids and total soluble carbohydrates with maturity. The carbohydrate concentration declined from 25% in immature seed to 10% in fully mature seed, indicating the use of carbohydrate in the production of lipids as the maturation advances. Electrophoretic studies of Yamada et al. (1980) and Basha et al. (1976) have shown that the storage protein, arachin, is rapidly accumulated during later stages of seed development, while nonarachin proteins are deposited early in the cotyledon development. Ten weeks after pegging, only 35% of the maximum amount of arachin had accumulated whereas 85% of conarachin II had accumulated. Arachin however, increased rapidly in the later stage of maturation. No change in the subunit ratio in arachin during seed growth was observed on the patterns of SDS electrophoresis (Yamada et al., 1980).

Differences in Growth and Partitioning Characteristics Among Peanut Genotypes

Genotypic differences have been observed in vegetative and reproductive growth characteristics of peanut lines differing in pod yield potential (Duncan et al., 1978). Periodic increments in plant dry matter can be expressed on a leaf area or land area basis, thereby allowing comparisons of relative production efficiencies of different plant

genotypes during varying growth stages (Pixley, 1985). With this information, partitioning of photosynthate to pod growth can be estimated (Duncan et al., 1978). Duncan et al. (1978) and Knauft and Gorbet (1990) estimated partitioning factors for several cultivars and showed that there are significant differences among various peanut genotypes in partitioning to reproductive tissue.

Penning De Vries et al. (1982) calculated the energy cost of producing different plant tissues. By using these production values calculated for different plant tissues, energy requirements for reproductive and vegetative tissue production can be obtained in terms of glucose equivalents. By using the glucose cost values of 2.556 for protein, 3.106 for lipid, 2.174 for lignin, 0.929 for organic acid, 1.242 for cellulose-carbohydrate and 0.05 for mineral, Boote et al. (1986) computed the synthesis cost for various peanut tissues. They estimated the cost of peanut seed with composition of 28% protein, 51% oil, 12.5% cellulose-carbohydrate, 2% lignin, 4% organic acid and 2.5% mineral as 2.54 g glucose g^{-1} of seed.

Pixley et al. (1989) calculated assimilate partitioning in terms of glucose equivalents by dividing the glucose equivalents of pod growth rate by the glucose equivalents of total CGR. The cost of total crop growth was the sum of CH_2O requirements for vegetative and reproductive growth. Using this procedure, Pixley et al. (1989) showed that

partitioning to reproductive growth is different among four cultivars differing in leafspot resistance.

Research Perspective

Three breeding lines, 529B, 563A and 647A, in the Florida peanut breeding program showed significant seed shriveling when harvested at full maturity. Also this character was observed to be expressed at different levels of intensity within a single true-breeding plant. Preliminary studies at the University of Florida indicated the shriveled seed in these lines contain only half to two thirds of normal oil concentrations. However, these plants produce a large number of pods and seeds per plant. So far there is no report of such seed morphology mutation in peanut that can also affect seed composition.

To understand the biochemical lesion associated with the shriveled-seed characteristic in peanut and thereby to study this locus at the molecular level, preliminary characterization of shriveled-seed mutation is very important. Placement studies are necessary to identify timing and location of different shriveled-seed phenotypes in the reproductive cycle of the plant because this character is observed to be expressed differentially on a single plant. Inheritance studies are important to understand the genetic control of the shriveled-seed characteristic. Chemical analyses of differentially

shriveled-seed types may be helpful to understand the differences in concentrations of various components in these seed and also to further investigate the accumulation patterns of various seed components at different developmental stages. Understanding the differences in the growth characteristics and partitioning of photosynthate to the pods in the shriveled-seeded lines vs. normal-seeded lines will be helpful to specify whether these differences exist in the vegetative phase of the plant or in the reproductive growth period.

With this rationale to characterize the shriveled-seed mutation, the present research was started with the specific objectives of studying (1) the placement of the shriveled-seed trait on an individual plant, (2) chemical composition of various phenotypic classes of shriveled seed and changes in the shriveled seed composition during development, (3) inheritance of the shriveled-seed characteristic, and (4) differences in the partitioning of photosynthate to pod growth in the shriveled-seeded genotype vs. normal-seeded genotypes. Various studies were conducted to meet the above objectives and results are presented in the following chapters.

CHAPTER 2
PLACEMENT STUDIES

Introduction

The seed-shriveling characteristic in mature seed of peanut lines, 529B, 563A, and 647A which are characterized by low oil concentrations and increased levels of sucrose, (Chapter 4) is not expressed uniformly on an individual plant. Placement studies may help to map the spatial and temporal patterns of expression of this trait in the pegging zone of the shriveled-seed genotype. It may later be helpful to understand the developmental cues involved in the differential expression of this trait.

The developmental morphology of the virginia type peanut is suggestive of a well defined and predictable pattern of fruiting (Gupton et al., 1968). Gregory et al. (1951) clearly described the morphology of the virginia peanut. The epicotyl of the seed consists of one terminal and two cotyledonary axils. The central axis (main stem) develops from the terminal bud and is flanked by two opposite, lateral branches which arise from the respective cotyledonary axils. The central axis is always erect but may be relatively shorter than the lateral branches.

According to their branching order notation, the central axis of the plant is the *n* order. Branches arising from the *n* order are the *n* + 1 order and branches arising from *n* + 1 order axes are the *n* + 2 order, etc. In virginia type peanut all the lateral buds of the central axis are vegetative. The first cataphyllar node of *n* + 1 order branches is vegetative and the second node is occasionally reproductive. The *n* + 2 order branches occur as pairs of vegetative branches alternating with pairs of reproductive branches and the same trend follows for the branches of all orders.

The inflorescence in peanut is subpaniculate and it always occurs in the axils of the foliage leaves or cataphylls. They form a definite branching pattern with the vegetative branches but never occur at the same node with them. Each inflorescence bears three to several flowers. The flowers usually appear one at a time, daily or at intervals of several days (Gregory et al., 1951). Generally the flowering in virginia peanut progresses from the basal branch to the upper branches and from the base of the respective branches to their tips (Gupton et al., 1968).

Gupton et al. (1968) identified the peanut fruits that belong to specific growth periods by tagging the pegs which penetrated the soil within three successive time periods. Using this method they were able to map the positions of the fruit on the cotyledonary lateral of

virginia type peanut and related these positions to various pegging groups, defined by the time of peg penetration. The pegs of virginia type peanuts occur initially on the first reproductive branch of the cotyledonary lateral and successive pegging extends outward and upward in concentric circles from the first inflorescence on the lateral. Three well defined pegging groups, early, intermediate, and late, can be identified by their order of appearance on the laterals (Gupton et al., 1968).

Determination of physiological maturity of peanut based on internal or external physical, morphological and color characteristics of the hull, seed coat, and seed has been described by Pattee et al. (1974), and Williams and Drexler (1981). Usually peanut fruits are considered physiologically mature when the inner pericarp is completely brown and starts showing black splotches over at least half the pericarp. At this stage the seed coat begins to turn from dark pink to brown in color (Pattee et al., 1974). The changes detected in various seed components such as lipids, carbohydrates, and volatiles also indicated a logical physiological progression according to the physiological maturity index (Sanders et al., 1982).

All of the three shriveled-seeded lines used in this study are virginia botanical types, with characteristic sequential branching and absence of reproductive branches on the main stem. However, plants in these lines are

characterized with a large numbers of lateral branches, pegs and pods. Norden (1975) reported that high drying temperature and immaturity were the major factors causing shriveled kernels in peanut. As the shriveled-seeded lines are characterized by many immature pods at any time of harvest, care was taken not to confound the immature seed shriveling with the typical seed shriveling seen in the physiologically mature seed from these genotypes. In this study, the inner shell darkness was taken as the physiological maturity index for scoring the number of mature shriveled seed that were present in each pegging zone or tagging date studied. In the present investigation for the placement of the shriveled character on a shriveled-seed plant, two individual studies were conducted. The first study involved dividing each $n + 1$ lateral on the plant into four pegging zones by following the scheme suggested by Gupton et al. (1968) with some modifications. The second study involved periodic tagging of the pegs that had just entered the soil. Only one shriveled-seed genotype was studied here because this study was primarily designed to verify the legitimacy of the results obtained from the first experiment. Because the plants of shriveled genotypes contain larger numbers of lateral branches with many pegs and pods as compared to the normal-seeded genotypes, the mapping procedure suggested by Gupton et al. (1968) might not be quite adequate for delineating the various pegging

zones on these plants. However, if the peg tagging study also indicates similar results, tagging the pegs to locate the shriveling character on a shriveled plant can be avoided. This will facilitate an easy identification of the shriveled seed location for the recovery of the seed samples at different developmental stages for other types of investigations.

Materials and Methods

Experiment 1

Plant genotypes

Three shriveled-seeded genotypes, 529B, 563A, and 647A and one normal-seeded cultivar Sunrunner were used in this study. Plants were sampled from a larger experiment sown at the rate of 21 seed for each 6.1 m long row with a row spacing of 0.91 m, in a randomized complete block design, replicated four times in the summer of 1991 at the University of Florida Agronomy Farm at Gainesville, Florida. At 140 days after planting (DAP), ten healthy plants from each shriveled line and five plants from Sunrunner from each replication were carefully lifted from the ground using a shovel. Plants were washed, air dried, and each plant was divided into various groups of lateral branches for the pegging zone classification.

Classification of peg positions on the laterals into various fruiting zones

In this classification the main stem was designated as n, and the primary branches arising from the main stem were n + 1. The secondary branches arising from the n + 1 branches were designated as n + 2 types. The subsequent branches originating from secondary branches were tertiary (n + 3) types. Each plant was separated into four pairs of primary branches, the first pair was designated as n + 1 (1), comprising with the first pair of cotyledonary branches. The second pair, n + 1 (2), included the third and fourth n + 1 laterals, the n + 1 (3) pair included the fifth and sixth n + 1 branches, and the n + 1 (4) pair included the seventh and eighth n + 1 laterals. Each n + 1 branch was further divided into vegetative and reproductive branches. The reproductive branches originating from n + 1 branches were n2(R) type and those originating from the n + 2 vegetative branches were n3(R) type (Fig. 2-1).

Peg positions on each individual branch on each pair of n + 1 lateral branches were considered as identical for dividing each branch into different pegging zones. Each lateral was divided into four pegging zones and the pods produced on these positions were grouped into the respective zones. All pods produced outer to the positions designated for Zone 3 and the pods that were produced on the n + 1 laterals above the n + 1 (4) belong to Zone 4. Peg positions that belong to each zone from each n + 1 pair

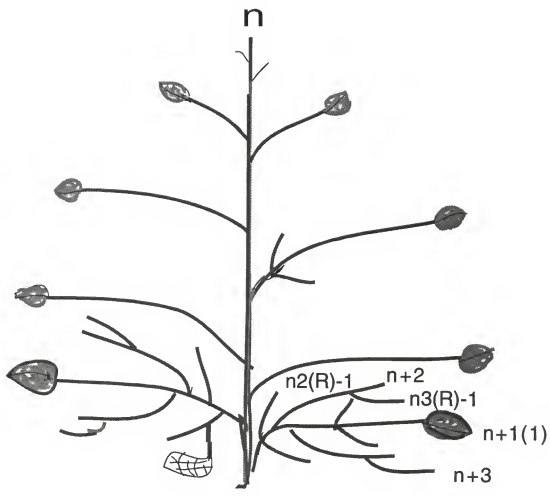

Figure 2-1. Classification of vegetative and reproductive branches based on their position on the main stem in a virginia type peanut.

are given in the Table 2-1. In this notation, for example, n2(R)-1-1,2,3 of n + 1 (1) represents the first, second, and third positions on the first pair of n + 2 reproductive branches on the first pair of n + 1 lateral. Similarly, n3(R)1-1 of n + 2 (2) of n + 1 (2) represents the first position on the first inflorescence of the n + 3 reproductive branch on the second pair of the n + 2 vegetative branch that belong to the second pair of the n + 1 branch. Figure 2-2 shows the peg positions that belong to various zones on an n + 1 (1) cotyledonary branch.

Determination of maturity and proportion of shriveled seed in each pegging zone of an individual plant

Pods that belong to each pegging group from each plant were dried separately, and each pod was hand shelled to assess physiological maturity, based upon the inner shell darkness. Only pods which at least showed the maturity characteristics of stage 11 and above (Pattee et al., 1974) were selected and all the other pods were discarded. The proportion of shriveled seed for each zone was obtained as the percentage of the total number of kernels present in that particular zone. The same procedure was also followed for the Sunrunner plants.

Statistical procedures for the placement of shriveled seed in the pegging zone of a shriveled-seeded plant

A multivariate repeated-measures analysis was performed using three shriveled lines (the between-line factor) and the proportions of shriveled seed across the pegging zones

Table 2-1. Classification of peg positions into four pegging zones based on their position on the lateral branches of the shriveled-seeded virginia type peanut plant.

Lateral on the Main stem	Position of the Pegs on Reproductive Branches			
	Zone 1	Zone 2	Zone 3	Zone 4
n + 1 (1)				
	n2(R) 1-1,2,3	n2(R) 1-4	n2(R) 2-3	All other positions on all levels of branches
	n2(R) 2-1	n2(R) 2-2		
		n2(R) 3-1		
n + 2 (1)				
	n3(R) 1-1	n3(R) 1-2,3	n3(R) 2-2	
		n3(R) 2-1	n3(R) 3-1	
		n4(R) 1-1	n4(R) 2-1	
		(of n + 3 (1))	(of n + 3 (1))	
n + 2 (2)				
			n3(R) 1-2	
			n3(R) 2-1	
n + 2 (3)				
			n3(R) 1-1	
n + 1 (2)				
	n2(R) 1-1,2	n2(R) 1-3	n2(R) 2-2	
		n2(R) 2-1	n2(R) 3-1	
n + 2 (1)				
	n3(R) 1-1	n3(R) 1-2	n3(R) 2-2	
		n3(R) 2-1		
n + 2 (2)				
		n3(R) 1-1	n3(R) 1-2	
			n3(R) 2-1	

Table 2-1 -- continued.

Lateral on the Main stem	Position of the Pegs on the Reproductive Branches			
	Zone 1	Zone 2	Zone 3	Zone 4
n + 1 (3)	n2(R) 1-1	n2(R) 1-2 n2(R) 2-1	n2(R) 1-3 n2(R) 2-2	
n + 2 (1)		n3(R) 1-1	n3(R) 1-2	
n + 2 (2)			n3(R) 1-2	
n + 2 (3)			n3(R) 1-1	
n + 1 (4)		n2(R) 1-4	n2(R) 1-2	
n + 2 (1)			n3(R) 1-1	

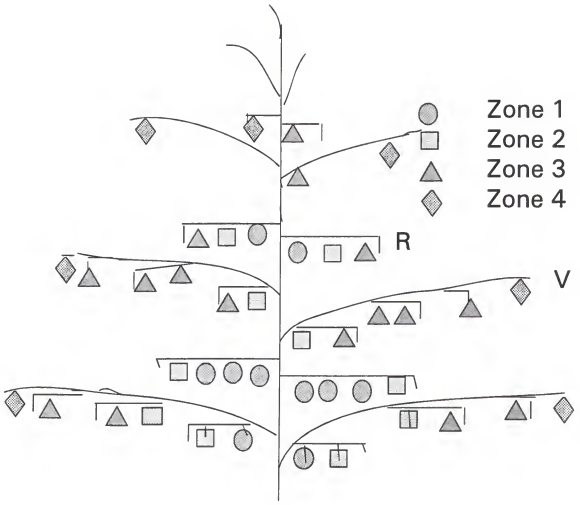

Figure 2-2. Peg positions representing four pegging zones on a n+1(1) cotyledonary branch.

(the within-line factor) using SAS General Linear Models program (SAS Institute, Inc., Cary, NC) with NOUNI Option. The percentage data was transformed using the arcsin transformation. The repeated measures analysis was employed in this analysis because the randomization of zones within the genotype was not possible.

Experiment 2

Plant genotypes

Four rows of shriveled-seeded line 529B and two rows of normal-seeded line Sunrunner were planted on May 19, 1992 at the University of Florida Agronomy Farm near Gainesville, Florida in rows 6.1 m long with a spacing of 0.91 m between rows and 0.30 m between seed within rows. Ten plants from the line 529B and five plants from Sunrunner were identified with colored flags and the pegs that had just started penetrating the soil were tagged with color coded surgical wound clips as previously described in Gupton et al. (1968) and Bennett et al. (1990). The first tagging was done on July 2, 1992 and nine more times with different color coded clips, at 5-day intervals, until August 22, 1992. By this time, most of the pegs had already penetrated the soil surface. At 150 DAP the soil around each plant was loosened and each plant was carefully lifted from the soil with a shovel, washed with water and air dried. Out of ten plants from the genotype 529B, five plants that showed loss of tagged pods and necrosis of branches due to disease were

discarded. Pods with a similar color code representing the same tag date were separated from each plant and grouped together. Pods were placed in small paper bags and oven dried at 60°C for four days. Pods from each individual plant and each individual tag date were hand shelled separately for determining the physiological maturity of the seed. The determination of physiological maturity and calculation of the percentage of mature shriveled seed for each tag date was done by following the same procedure as in the previous experiment.

Statistical analysis

A repeated measures analysis was done with five 529B plants as between-line factors and the shriveling across the tag dates as within-subject factor, using the SAS GLM program (SAS Institute, Inc., Cary, NC).

Results and Discussion

Effect of Pegging Zone on the Shriveled Seed Expression

The multivariate repeated measures analysis revealed that the four pegging zones have different multivariate means for the amount of shriveling expression, as indicated by the likelihood test statistic (P=0.0001) (Table 2-2). The lack of Zone*Line interaction (P=0.25) between the zones

Table 2-2. Analysis of variance for multivariate repeated measures analysis to test within line effects using maximum likelihood statistic (Wilks' Lambda).

Source of Variation	Wilks' Lambda Value	Approx. F Value	P Value
Zone	0.15704	189.65**	0.0001
Zone*Line	0.19813	1.66	0.2473
Zone*Block*Line	0.81738	1.23	0.2332

** Significant at 0.01 level of probability

and lines (Table 2-2) (Fig. 2-3) indicated that the differences in the amount of shriveling may not depend on the different shriveled lines used. The analysis of variance for the shriveled lines showed no significant difference for the amount of shriveling among the three lines tested (Table 2-4). The polynomial contrasts presented in Table 2-3 showed significant linear trends for the amount of shriveling across zones. The mean percentage for shriveled seed increased from Zone 1 to Zone 4 in all of the shriveled-seeded lines (Table 2-5) indicating that the seed produced on the outer pegging positions has more tendency for the expression of shriveled characteristic compared to the inner zones. No shriveling has been observed in any of the zones in the mature seed of Sunrunner.

Effect of Pegging Time on the Shriveled Seed Expression

The repeated measures analysis for five 529B plants for ten peg tagging dates indicated that the amount of shriveling was significantly different among different tag dates (P=0.0001). However, there were no differences among the five shriveled-seeded plants for this expression (Table 2-6). The mean percentage of shriveling for each tag-date indicated that the amount of shriveling had increased from the first tag-date to the 8-10 tag-dates (Table 2-7)

Table 2-3. Analysis of variance for the polynomial contrasts for zones.

Source of Variation	Degrees of Freedom	Mean Square	F Value	P Value
Zone (Linear)	1	10.65	504.04**	0.0001
Zone (Quadratic)	1	0.01	0.63	0.4308
Zone (Cubic)	1	0.03	1.58	0.2109
Error	108	0.02		

** Significant at 0.01 level of probability

Table 2-4. Analysis of variance for between-line effect for three shriveled-seeded lines.

Source of Variation	Degrees of Freedom	Mean Square	F Value	P Value
Line	2	3.67	10.68*	0.0105
Error (Block*Line)	6	0.34		

* Significant at 0.01 level of probability.

Table 2-5. Effect of pegging zone on the percentage expression of shriveled seed.

Shriveled-Seeded Genotype	Zone 1		Zone 2		Zone 3		Zone 4	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	----------				% ----------			
529B	70.5	1.5	76.9	1.5	85.2	1.2	92.3	1.1
563A	76.1	1.6	86.0	1.5	90.7	1.1	94.4	1.0
647A	58.5	2.5	66.0	2.7	72.0	2.6	80.75	2.2

SE = Standard error of mean

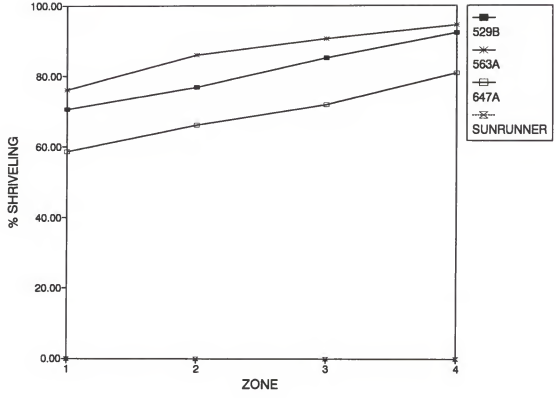

Figure 2-3. Effect of pegging zone on the shriveled seed expression.

Table 2-6. Analysis of variance for the repeated measures analysis of peg tagging dates.

Source of Variation	Degrees of Freedom	Mean Squares	F Value	P Value
Plant	4	176.90	0.89	0.4794
Tag-date	9	2447.14	12.32 **	0.0001

** Significant at 0.01 level of probability

Table 2-7. Effect of pegging time on the expression of shriveled seed of line 529B.

Plant #	Percentage of Seed Shriveling									
	Tag-date									
	1	2	3	4	5	6	7	8	9	10
	%									
1	50.0	52.4	16.7	50.0	71.4	57.7	93.3	92.3	100.0	96.2
2	44.4	51.8	33.3	50.0	58.3	63.6	75.0	84.6	92.8	77.8
3	25.0	47.6	57.1	50.0	36.4	63.6	80.0	100.0	62.5	65.0
4	40.0	26.7	60.0	17.7	54.5	33.3	83.3	77.7	100.0	100.0
5	33.3	37.5	58.8	33.3	50.0	83.3	71.4	100.0	100.0	100.0
Mean	38.7	44.7	45.2	40.2	54.1	60.3	80.6	90.9	91.1	87.8
SE	4.8	5.4	8.7	6.5	5.7	8.0	3.8	4.4	7.3	7.0

SE = Standard error of mean

Fig.(2-4). The normal-seeded cultivar did not show seed shriveling in the mature seed for any of the tag dates.

Both of these experiments clearly indicated that more shriveled seed are produced in the outer pegging zones or in the later tag-dates. Gupton et al. (1968) showed that the pegging begins at inflorescence positions on the lateral near the main stem and progresses outward to positions on both reproductive and vegetative branches of all orders. The peg development also occurs in essentially the same order as peg placement. Although the shriveled seed were present in all of the pegging zones and in almost all of the tag-dates, there was an increasing trend for their appearance in the later-formed pods. In other words, shriveling was more pronounced on higher order reproductive branches than on the second order reproductive branches, on which most of the first formed pods are present. Similar results were reported in the soybean shriveled-seed mutant, 328-1 (Honeycutt et al., 1989). More shriveled seed were produced on the upper nodes than at the lower nodes. Even though this type of differential expression of a seed phenotype cannot be explained by the present knowledge of developmental control mechanisms of the plant, it is obvious that there is a correlation between the shriveling expression and basic plant developmental control.

While investigating the expression of the maize transposable element *suppressor-mutator (spm)*, Fedoroff and

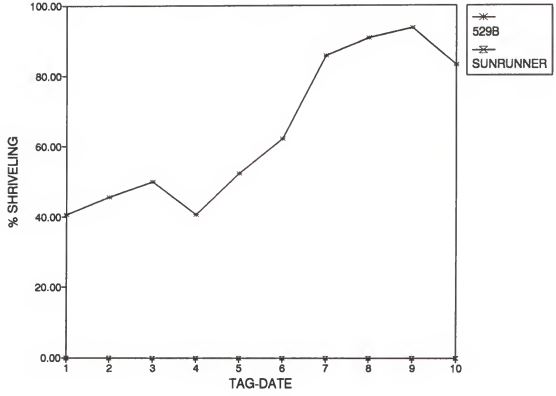

Figure 2-4. Effect of pegging time on the shriveled seed expression in line 529B.

Banks (1988) provided a clear evidence for the involvement of basic developmental regulatory mechanism of the maize plant in its different temporal or spatial patterns of expression during the development of the plant. Their study indicated that even though the inactive spm is transmitted to the ears of a single plant, the expression was differential in the main stalk and the tillers. While the spm is inactive in the main stalk, it was reactivated in the tillers of a single plant. Bhattacharyya et al. (1990) showed that the wrinkled pea expression is due to a transposon-like insertion in a gene encoding for the starch-branching enzyme. A transposable element can exist in one of three states. The three states for maize spm are a constitutively-active state in which the element is expressed throughout development, a cryptic state in which the element is genetically undetectable, and the third state where the element is programmed to be expressed in any one of many different temporal or spatial patterns during the development of the plant (Fedoroff and Banks, 1988). If the seed shriveling expression in peanut is caused by a transposon-like element, as in the case of wrinkled pea (Bhattacharyya et al., 1990) there might be a possibility that both the shriveled phenotype and its differential expression are essentially the state of expression of single element, controlled by fundamental developmental mechanism of the plant.

CHAPTER 3
INHERITANCE OF THE SHRIVELED SEED TRAIT

Introduction

Seed mutations affecting both seed morphology and composition are present both in cereals and legumes. Maize (*Zea mays* L.) kernel mutants brittle-1 (bt-1), shrunken-2 (sh-2) and miniature (mn) having shrunken or collapsed phenotypes and decreased levels of starch exhibit single gene inheritance (Mangelsdorf, 1926; Lowe and Nelson, 1946). All the shrunken endosperm mutants described in barley (*Hordeum vulgare* L.) by Jarvi and Eslick (1975) are inherited as monofactorial recessives with the exception of the mutant *se*6 that is inherited as a monofactorial recessive expressing xenia. Wrinkled pea (*Pisum sativum* L.) mutations described by Kooistra (1962) are controlled by single recessive genes. A recently described shriveled-seed mutation in soybean (*Glycine max* L. Merr) (Honeycutt et al., 1989a) is also monogenic in inheritance. Molecular analyses of Bt-1, and Sh-2 in maize and the R locus in pea showed that each of these loci encode a major enzyme/enzyme subunit in the starch biosynthetic pathway, supporting the classical idea of monogenic inheritance (Bhave et al., 1990; Sullivan et al., 1991; Bhattacharya et al., 1990).

Nonetheless, in all these mutants the shrunken expression is uniform within the genotype except for the soybean shriveled mutation, where the shriveled locus showed different levels of expressivity within a single plant (Honeycutt et al., 1989a).

Seed shriveling that severely affects seed morphology and the amount of lipid deposition in peanut is a unique type of seed mutation in peanut germplasm. Studying the inheritance of this character may be helpful for further understanding genetic control of this trait and the phenotypic changes that this mutation causes in the seed morphology and composition. Understanding the mode of inheritance of this trait will help to gain further insights into the poorly understood system of regulation of lipid biosynthesis in plant tissues (Slabas and Fawcett, 1992). Agronomic interest also lies in the genetic studies of this trait, because the low oil nature of this mutation may be helpful in the development of peanut breeding lines for naturally low calorie peanut snack foods. Differential expression of this trait on an individual true-breeding plant may also be explained by understanding the genetic regulation of the gene(s) controlling this trait.

The main objectives of the present research are: (1) to make both direct and reciprocal crosses between the shriveled-seeded types and normal-seeded type to determine the inheritance of the shriveled-seed character, and (2) to

make crosses among the shriveled-seeded lines in all possible combinations to determine the allelic relationships.

Materials and Methods

Three shriveled-seeded lines 529B, 563A and 647A and a normal-seeded cultivar Sunrunner were used in this study. As described later in Chapter 4, the degree of shriveling in the lines 529B and 563A was more severe compared to the moderate shriveling in 647A. In the spring of 1991, crosses were made in both directions, using shriveled lines as male and female parents. Crosses were also made among all the shriveled lines. F_1 seed from all crosses were planted in the greenhouse in the winter of 1991 to obtain the F_2 seed. The F_2 seed was field planted at the University of Florida Green Acres Agronomy Farm near Gainesville. The soil type was Arredondo fine sand. On 19 May, 1992, seed from each F_1 plant was planted in two row plots, at the rate of 21 seed per each row. Each row was 6.1 m long with 91 cm distance between the two rows. Identity of F_2 plants from individual F_1 plants were maintained for each cross. After 145 DAP, plants from each row were harvested separately and each plant was arranged in drying sheets in such a way that they could be easily separated after drying. Plants were dried in tobacco driers at 40 °C for about 1 wk. Each individual plant was threshed and shelled separately. Classification of F_2 plants for seed shriveling was determined based upon

the expression or non-expression of shriveling characteristic (see Chapter 4 for the description of typical kernel characteristics of shriveled seed) on F_3 seeds.

F_3 progenies were raised from selected F_2 plant families from each cross. The selection was mainly based on the number of plants in the F_2 family and number of F_3 seed produced on each plant. In each individual cross, families with more plants and individual plants with more seed were selected for F_3 planting. Only the plump seed from shriveled F_2 plants were selected for planting to give F_3 progenies. F_3 seed were planted on 5 May, 1992 with the same field specifications used for the F_2 generation at Green Acres Agronomy Farm near Gainesville. Plants were harvested on 29 September, 1993, and the seed phenotype of each individual F_3 plant was determined on F_4 seed in the same way described for F_3 seed.

Results

Crosses with Shriveled Lines as Male Parents

Even though very few F_1 seed were obtained from each cross, none of them expressed the characteristics of the shriveled-seed phenotype. All of the F_1 seed from each individual cross were plump and appeared normal. Mature seed harvested from the greenhouse grown F_1 plants from each cross contained a mixture of normal and shriveled-seed

phenotypes, indicating segregation for this characteristic in the F_2.

Crosses with 529B as male parent

Nine F_2 families were grown from the F_2 seed harvested from nine F_1 plants. All nine families showed a segregation into shriveled and non-shriveled types. The pooled F_2 plant data (Table 3-1) indicated that the normal to shriveled-seed phenotypes were segregating at a 3 normal : 1 shriveled ratio. Out of these nine F_2 plant families, three larger families, well represented by both shriveled and normal plants, were selected for advance to F_3. Nine plants from each of the three families were selected and a total of twenty-seven F_3 progeny rows were planted. Progenies from normal F_3 seed were expected to produce either true-breeding normal-seeded lines or lines that segregate for normal and shriveled types in a 1:2 ratio. The shriveled lines were expected to produce only true breeding recessive homozygous shriveled F_3 progeny. Out of twenty four non-shriveled normal-seeded plant families, nine families did not show segregation, while fourteen families segregated for normal and shriveled types in a 3 normal : 1 shriveled ratio (Table 3-2). All four of the shriveled-seeded lines produced only shriveled F_3 progeny. These results indicated that a single recessive gene is responsible for the expression of the shriveled-seed character in the cross Sunrunner X 529B.

Table 3-1. Segregation of shriveled-seed character in F_2 progeny (data obtained on F_3 seed) from the crosses between Sunrunner and shriveled-seeded male parents.

Cross	F_2 Seed Phenotype			X^2 (3:1)	P
	Total	Normal	Shriveled		
Sunrunner X 529B	131	98	33	0.0025	0.96
Sunrunner X 563A	36	26	10	0.1481	0.70
Sunrunner X 647A	116	94	22	2.2529	0.13
Pooled	283	218	65	0.6231	0.43
Homogeneity				2.4035	0.50

Table 3-2. Segregation of shriveled-seed character in F_3 progeny (data obtained on F_4 seed) from the crosses between Sunrunner and shriveled-seeded male parents.

Cross	Non-segregating Normal	Segregating 3 Normal: 1 Shriveled	Non-segregating Shriveled	X^2 (2 Segregating : 1 non-segregating)	P
Sunrunner X 529B	9	14	4	0.35	0.56
Sunrunner X 563A	8	6	3	7.71	0.06
Sunrunner X 647A	8	6	4	7.71	0.06

Crosses with 563A as male parent

Only three F_2 plant families were possible from the three F_1 plants from the cross Sunrunner X 563A. F_2 segregation ratio on the F_3 seed fit a 3:1 ratio for normal to shriveled-seed types (Table 3-1). When a total of seventeen F_3 progeny derived from seventeen F_2 plants were scored for the shriveling trait, from fifteen normal F_2 plants, eight bred true and six F_3 progeny showed segregation which obviously did not fit a 2:1 segregating to non-segregating ratio (Table 3-2).

Crosses with 647A as male parent

A total of ten F_2 plant families were grown from F1 plants. F_2 plants as classified from the F_3 seed segregated into 3 normal : 1 shriveled types (P=0.13). Out of 116 F_2 plants, 94 were normal and 22 showed shriveled phenotype (Table 3-1). Similar to the case of the previous cross with 563A as a male parent, eight F_2 families did not segregate but six showed segregation. This did not fit a 1:2 non-segregating to segregating family ratio (P=0.06) (Table 3-2).

Crosses with Shriveled Lines as Female Parents

While the crosses with shriveled lines as paternal parents segregated in a simple mendelian fashion, the reciprocal crosses with all three shriveled lines and the normal male parent produced all normal F_1, F_2 and F_3 seed progenies (Table 3-3). However, some of these families

Table 3-3. Phenotype of F_2 progeny (data obtained on F_3 seed) from crosses between shriveled-seeded lines as the female parents and Sunrunner as the male parent.

Cross	No. of F_2 Families	No. of F_2 Plants	Normal	Shriveled
529B X Sunrunner	8	97	97	—
563A X Sunrunner	10	94	94	—
647A X Sunrunner	5	49	47	2

Table 3-4. Segregation of shriveled-seed character in F_3 progeny (data obtained on F_4 seed) from crosses between shriveled lines as the female parent and Sunrunner as the male parent.

Cross	No. of F_3 Families	
	Non-segregating (Normal)	Segregating (Normal : Shriveled)
529B X Sunrunner	10	2
563A X Sunrunner	7	5
647A X Sunrunner	5	7

showed a segregation in the F_3 progeny (Table 3-4) based on classification of F_4 seed. These data did not fit a 1:2 non-segregating to segregating ratio, and the offspring in the segregating families did not fit a monogenic ratio.

Discussion

Crosses with Shriveled Lines as Male Parents

The cross Sunrunner X 529B satisfactorily explained that the shriveled-seed character is a recessive, monogenically inherited trait. As the chemical characterization of the mutant (Chapter 4) showed a severe reduction in the important seed components, storage lipid, certain subunits of storage protein, and elevated levels of sucrose, there appeared to be a pleiotropic association between the shriveled locus and both seed morphology and composition or there is a possibility that the shriveled locus is a regulatory locus controlling the expression of all these traits.

Because the cultivated peanut is an allotetraploid that behaves like a diploid (Smart and Stalker, 1982), most of the traits are expected to be digenically inherited unless the locus in question is mutated for some other function or has become a null allele. Nevertheless, a few simply inherited characters associated with seed composition in peanut have been reported: a high oleic acid character (Moore and Knauft, 1989) and two independent genes

responsible for the expression of two different sub-units of arachin protein (Krishna et al., 1986). However, none of these mutations affected the seed morphology. Thus this seed morphology mutant identified in the University of Florida peanut breeding program will be the first seed mutation discovered in peanut which drastically affects seed lipid concentration and sucrose levels, thereby giving a shriveled-seed phenotype. This shows a close resemblance with other very well characterized mutations like ra and rb loci in pea (Edwards et al., 1988; Smith et al., 1989) and bt-1 and sh-2 (Tsai and Nelson, 1966) in maize which mainly affect starch biosynthesis and give shrunken seed phenotype.

The observed ratio tested to fit the F_3 segregation of normal F_2 plant progenies of both crosses, Sunrunner x 563A and Sunrunner X 647A did not fit the expected 2 : 1 segregating to non-segregating ratio. This may be because the smaller family size associated with poor germination, which was about 50% in both crosses and may have reduced the survival of shriveled plants. However, the most important reason must be because of too few seed produced on some plants, which badly interfered with the scoring. As described in Chapter 2, the shriveled character does not show a complete penetrance, and more importantly, its expression starts later in the reproductive cycle of the plant. Most of the seed that are produced earlier in the reproductive phase will be normal. In this situation, plant

phenotype could be misidentified as a normal type if insufficient shriveled seed are produced on a shriveled type plant. Also the potential for interaction of shriveled genotype with the normal cytoplasm cannot be eliminated as the possibility for the reduced penetrance of the shriveled trait. The stringless pod character that has been described recently by McGee and Baggett (1992) in pea also showed varied F_2 ratios among crosses with stringy lines and the populations always had more stringy plants than expected. The ratio of 1 non-segregating (stringy): 2 segregating F_3 families derived from F_2 plants fit only for few families. They suggested that poor competitive ability of pollen bearing the stringless factor was the reason for deficiency of stringless plants.

Backcrossing the F_1s to the shriveled parent and studying the BC_1F_1 segregation may help to confirm the above results. Testing F_3 progeny segregation using larger F_3s for the crosses with 563A and 647A as male parents may provide additional strength to the conclusion that the shriveled trait is controlled by a single recessive gene.

Crosses with Shriveled Lines as Female Parents

While the crosses with shriveled lines as paternal parents segregated in a simple mendelian fashion, the reciprocal crosses involving all three shriveled lines with the normal male parent yielded all normal F_1, F_2 and F_3 seed

progenies. This may not be attributed to the transmission and exclusive expression of paternal extrachromosomal factors like plastids or mitochondria since these crosses showed segregation in a few families in the F_3 progeny. Also, an F_2 genotype with a homozygous shriveled nuclear makeup with the normal (paternal) cytoplasm should give a shriveled phenotype, as in the case of reciprocal crosses (shriveled homozygous recessive). However, there were no shriveled phenotypes observed in the F_2 generation.

Paternal inheritance in peanut (*Arachis hypogaea* L.)

So far there are only two reports of paternal inheritance of characters in peanut. The isozyme glutamate oxaloacetate transaminase (GOT) pattern in the F_1 progeny of eleven crosses (including reciprocals) reported by Grieshammer and Wynne (1990) and X-ray induced foliaceous stipule character expression in the F_1 (Mouli and Patil, 1975) showed strict paternal expression. However in both these cases these characters segregated in a normal mendelian fashion in F_2 indicating the expression in the F_1 was of the recessive characteristic. Neither report provided a satisfactory explanation for their observations but Mouli and Patil (1975) by examining their F_2 ratios, concluded that the paternal transmission of foliate stipule is under the control of two recessive genes transmitting through the male parent.

Paternal transmission of plastids and mitochondria as a source for character expression

Other than gymnosperms (Hagemann, 1993), there are only one or two cases of complete inheritance of paternal plastids in higher plants (Tinley-Bassett and Birky, 1981; Masoud et al., 1989; Shumann and Hancock, 1989). Even though evidence exists for the entry of many paternal mitochondria into the egg cell during fertilization (reviewed by Connett, 1989), there is no experimental evidence for the expression of biparental or paternal mitochondrial expression in higher plants except for the latest report in rapeseed (*Brassica napus*) by Erickson and Kemble (1993). Corriveau et al. (1988) screened 200 angiosperm species to detect biparental plastid transmission using fluorescein microscopy and concluded that there was no plastid transmission through pollen in the species *Arachis hypogaea* L.

Genomic imprinting as a possibility for the lack of shriveled expression in shriveled X normal crosses

If the paternal or biparental transmission of extra nuclear genes cannot explain the possibility for the non-mendelian expression in the reciprocal crosses, an alternative phenomenon that might operate in this special case could be due to heritable changes the normal allele caused in the structure or expression of the shriveled allele while both were in the heterozygote.

Both the organization of chromatin and DNA methylation processes are involved in the regulation of gene expression. Also it is highly likely that different methylation patterns also correspond to differences in chromatin conformation (Meyer 1993a). In the well-studied phenomenon of paramutation in the two unlinked duplicate loci in maize, R (Brink et al., 1968) and B (Coe 1966), the paramutable allele changes its expression following passage through a paramutagenic allele in the heterozygote. The change in the expression of the paramutable allele was heritable even a few generations after it emerged from the heterozygote, until it recovered its former activity over several sexual generations. The paramutational changes at the R-locus correlate with changes in the degree of methylation of the R-allele (Dooner et al., 1991). In other words, the allelic interaction in the heterozygote can cause heritable changes in the expression of the paramutable allele. Even though the paramutant allele is responsible for the reduction in the expression of the paramutable allele in all the above cases and also in a recent study in a flower color gene expression in transgenic Petunia (Meyer, 1993b), by principle, the paramutation phenomenon shows how effectively the paramutant allele can bring a change in the expression of its counterpart while interacting with it in the heterozygote.

Similarly, imprinting of shriveled gene(s) and preferential expression of paternal alleles due to epimutational changes that might have taken place in these alleles may account for the expression of normal-seeded types. Imprinting is obviously heritable and evidence has been obtained for transmission of altered methylation patterns through the germline (Holliday, 1987). As imprinting is reversed in the germline, possibly at the time an inactive allele is reactivated. There are many examples of this phenomenon both in animals and plants (reviewed in Matzeke and Matzeke, 1993). It is suggested that homologous regions of the genome communicate and compare the epigenetic modifications they have undergone. The 'cross talk' enables homologues to compare their degree of relatedness. Irregularities observed between homologous alleles may result in 'repair ', e.g., mitotic recombination, gene conversion etc., or differential expression of the alleles concerned. The outcome may be preferential maternal or paternal allele expression or cellular mosaicism (Monk, 1990).

Another phenomenon which is known as position effect variegation in Drosophila refers to the mosaic phenotype which results from an inactivation of gene expression in some cells due to an inhibitory *cis* effect of an adjacent heterochromatic block. This effect includes modification of chromosome structure and seems to be dependent on the amount

of heterochromatin present (Dimitri and Pisano, 1989). We may consider the differential expression of seed shriveling characteristic in shriveled-seeded lines as comparable with position effect variegation phenomenon and under the influence of flanking heterochromatin regions. When the shriveled line is crossed with a normal line, during meiosis, the allele may be placed in an euchromatic environment due to crossing over and recombination events, resulting in normal-seed expression. It has been proposed that the recombination at meiosis may play an essential role in the repair of epigenetic defects. Several observations on crossing over and gene conversion in eukaryotes are relevant to this possibility (Holliday, 1987). The uneliminated or uncorrected epigenic defects will be transmitted to the next generation. With this argument, in the successive generations of shriveled X normal crosses, there will be an appearance of shriveled types as the population becomes more and more homozygous upon repeated selfing.

Crosses Among All Shriveled Combinations

The three shriveled lines were crossed in all possible combinations to determine the allelic relationships. Except for the cross 529B X 563A which was missing, all other cross combinations (Table 3-5) showed no segregation for normal condition in the F_2 generation (on the F_3

Table 3-5. Segregation of shriveled-seed character in the crosses among the three shriveled lines.

Cross	F_1 Seed Phenotype	F_2 Seed Phenotype		
		Total	Normal	Shriveled
563A X 529B	Shriveled	139	--	139
563A X 647A	Shriveled	87	--	87
647A X 563A	Shriveled	101	3	98
647A X 529A	Shriveled	66	--	66
529B X 647A	Shriveled	37	--	37

seed) indicating that all the shriveled loci in these genotypes were allelic.

In conclusion, the shriveled-seed character in peanut should be further examined for all the ambiguities uncovered in the present research, by crossing the shriveled line with several normal lines and by generating iso-cytoplasmic lines of both shriveled and normal parents and crossing them in both directions. These types of experiments can eliminate the possibility for paternally transmitted organellar expression as a reason for the paternal inheritance of this trait. Once the possibility for extra-nuclear inheritance is eliminated, further investigations on cytological behavior and molecular basis for this phenomenon can be investigated.

CHAPTER 4
CHEMICAL COMPOSITION OF SHRIVELED SEED MUTANTS IN PEANUT

Introduction

Peanut is grown for its high quality vegetable oil and production of various food products like peanut butter, roasted and salted nuts and candy. Even though reports from ICRISAT or elsewhere (Dwivedi et al., 1990; Salunkhe et al., 1992) show that the oil content in peanut seeds ranges from 36 - 56%, and protein content from 16.2 - 36%, the oil content in most of the commercially grown cultivars averages around 50% and the protein concentration averages around 25% (Salunkhe et al., 1992; Dwivedi et al., 1990). Limited existing genetic variability, limited research on artificial induction of mutations to change seed composition and very little information that is available today on biotechnological approaches for the alteration of peanut seed composition may limit some of the potential uses of peanut in specialty food markets.

Most of the studies conducted on peanut kernel composition show that changes in composition during kernel development occur in the three major components, lipid, protein and carbohydrates. Peanut oil is usually composed of 96% triacylglycerols, 2% diacylglycerols and about 1%

polar lipids (Sanders, 1980). The triacylglycerols increase from 85% in the immature stage to 96% in the mature stage and polar lipids and free fatty acids decrease with maturity. For total protein, the globulin fraction accounts for 87%, with two types of proteins, arachin and conarachin predominating (Basha and Pancholy, 1981). Arachin exists as a monomer as well as a dimer. However, both the monomer and dimer consist of three kinds of polypeptides of about 60 kd which associate to make a holoprotein of 180 kd (monomer) and 360 kd (dimer) (Yamada et al., 1979). Conarachin is a complex protein consisting of one kind of polypeptide (Yamada et al., 1980). Yamada et al. (1980) and Basha et al. (1976) have shown that arachin rapidly accumulated during later stages of seed development while nonarachin proteins are deposited early in cotyledon development. No change in the subunit ratio in arachin was observed during seed growth, as revealed by patterns of SDS electrophoresis (Yamada et al., 1980). The total carbohydrates in peanut seed amount to about 32-38% of the defatted meal; of this, the mono and oligosaccharides account for 18%, of which 13.9% is sucrose. Starch makes up 12.3% (Taranathan et al., 1975). While studying the compositional changes in peanut kernels, Pattee et al. (1974) showed that starch reaches a maximum just beyond middle maturity stage of the seed and remains constant, whereas sugar content increases throughout maturation, reaching a maximum at full maturity.

Three peanut breeding lines, 529B, 563A and 647A identified in the Florida peanut program, show seed shriveling even after harvested at full maturity. As described in Chapter 2, the expression of shriveling was not uniform on a single homozygous true-breeding plant. These mutant genotypes can produce seed with different levels of shriveling, resulting in seed phenotypes ranging from normal round seed to mostly shriveled seed on a single plant. Although the same genetic locus is controlling the shriveling characteristic in all these shriveled genotypes (Chapter 3), the degree of penetrance of this mutation is different among the three genetic backgrounds. Therefore, the appearance and the proportion of each shriveled-seed phenotype is different, depending upon the genetic background.

All other seed morphology mutations described in other crop species are associated with changes in one or more major seed chemical components. The wrinkled pea types described by Kooistra (1962), maize shrunken kernel mutations described by Creech (1965), and barley endosperm mutants described by Jarvi and Eslick (1975) all have deficiency of a major enzyme in the starch metabolic pathway. The shriveled-seed mutant described in soybean was found to have reduced expression of a sub-unit of storage protein, ß-conglycinine (Honeycutt et al., 1989). Preliminary chemical analyses of shriveled peanut seed from

various shriveled-seed phenotypes indicated there were differences in the concentration of lipids, protein and sugars.

The primary goal of this study was to characterize different shriveled-seed phenotypes for all major seed chemical components. A developmental comparison of the shriveled-seeded phenotype with a normal phenotype was performed after establishing the differences in chemical composition of shriveled and normal seed.

Two experiments were conducted for studying the chemical composition of different shriveled-seed phenotypic classes. The first experiment involved the estimation of various seed components for all phenotypic classes in all three mutant genotypes and in the normal-seeded type, Sunrunner. The second experiment was conducted to examine the lipid and protein profile of the various shriveled phenotypic classes of the shriveled genotype 529B and Sunrunner. The third study was a developmental study involving shriveled type and normal type seed at four developmental stages, immature, late immature, intermediate and mature stages sampled only from the shriveled genotype, 529B. The lipid and protein profile of both shriveled and normal-seed types were compared at the four developmental stages.

Materials And Methods

Experiment 1

Seed composition study

This experiment was planted on May 5, 1991 at the University of Florida Green Acres Agronomy Farm, Gainesville, FL. The soil type is an Arredondo fine sand. Three true-breeding shriveled-seeded genotypes (529B, 563A and 647A) and a normal-seeded cultivar Sunrunner were planted in a randomized complete block design, with four replications at the rate of 21 seed per each 6.1 m of row. The row spacing was 91 cm. Standard cultural practices were followed throughout the growing season. At 140 DAP sufficient number of plants from each genotype from each replication were harvested and dried. From each genotype only the seed from fully mature pods, based upon their inner shell darkness, were collected and the remaining immature seed were discarded.

Classification of mature shriveled seed into various phenotypic classes

The seed obtained from the mature pods from each shriveled-seeded genotype were a mixture of plump seeds and shriveled seeds with various levels of shriveling. Seed from each genotype were subjectively divided into five phenotypic classes with Class 1 as the most shriveled type and Class 5 as the normal round type (Figures 4-1 and 4-2).

Figure 4-1. Five phenotypic shriveled-seed classes of 529-B.

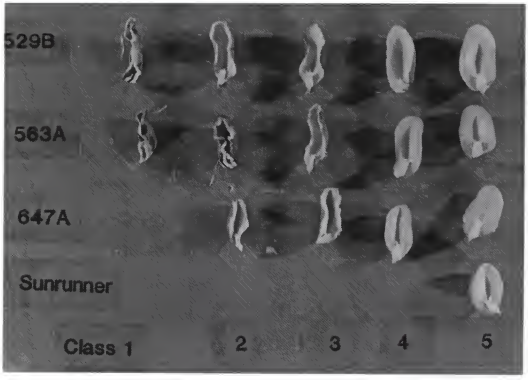

Figure 4-2. Longitudinal sections of various shriveled-seed phenotypic classes from three shriveled-seeded lines, 529-B, 563-A and 647-A and Sunrunner.

Class 2 is little less shriveled compared to Class 1 and Class 3 is intermediate in shriveling and Class 4 seed was characterized by a dent on the seed (Figure 4-1). The seed were somewhat concave inside the cotyledons, depending upon the level of shriveling of the kernel (Figure 4-2). According to this classification, the seed from line 529B and 563A were divided into five phenotypic classes and the line 647A was divided into only three classes, Class 3, 4 and 5. The shriveling expression was less intense in this genotype with no Class 1 and 2 seed types being produced.

Each class of shriveled seed from shriveled-seeded genotypes, and mature seed from the normal-seeded Sunrunner line were analyzed for concentration of total lipid, total protein, sucrose, starch, mineral and moisture. There were four field replications.

Lipid extraction

A modified Folch extraction procedure with dichloromethane and methanol was used for the total lipid extraction from 3 g of each shriveled-seed class (Christie, 1982). Dichloromethane and methanol were used in a 2:1 ratio, at a proportion of 1:10 (w/v) for the sample to the solvent. Seed were first homogenized in methanol for 1 min in a blender and dichloromethane was added and again homogenized for 2 min. The extract was filtered and the cake was re-extracted by repeating the same procedure. The filtrate was measured and transferred into a separatory

funnel and 0.88% KCL in water, 25% to the total volume of the filtrate was added and the mixture was thoroughly shaken and allowed to separate for 8 hr. The lower lipid-containing layer was removed into a round-bottomed flask and the solvent was evaporated using a Buchi rotary evaporator. The lipid concentration was reported gravimetrically on a dry weight basis.

Protein estimation

Protein was estimated as total nitrogen which was converted into crude protein by multiplying with a factor of 5.46 (AOAC, 1984). Nitrogen analysis was done by using a modified Kjeldahl procedure. Samples were first defatted two times using petroleum ether and the meal from each sample was digested using a modification of the aluminum block digestion procedure of Gallaher et al. (1975). The digestion was conducted on 300 mg of each sample using 3.2 g of 9:1 K_2SO_4:$CuSO_4$ catalyst, 10 mL H_2SO_4 and 2 mL H_2O_2 for 4 hr at 400°C. The ammonia in the digestion product was determined by semiautomated colorimetry (Hambleton, 1977).

Sucrose estimation

Sucrose in the defatted meal from each sample was estimated using the resorcinol procedure of Roe (1934). Samples were defatted twice using petroleum ether. Approximately 75 mg of finely ground defatted meal of each sample was boiled in 80% ethanol for 1 hr and the extract was adjusted to a final volume of 25-30 mL and the volumes

were measured. A 300 μL aliquot of the extract was placed into a test tube and 0.7 mL of water and 1 mL of 1 N NaoH was added and vortexed. The tubes were boiled for 10 min and then allowed to cool to room temperature. This procedure destroys the free reducing sugars. To each tube 1 mL of 0.1 % resorcinol reagent in ethanol and a 7 mL of 9 N HCl was added and again heated at 80°C for 8 min. The tubes were cooled and vortexed and the absorbance was read at 520 nm. Standard solutions of fructose with various concentrations ranging from 15 μg to 150 μg mL^{-1} were made and from each standard, 100 μL aliquot was transferred into a test tube and 1.9 mL water was added and vortexed. The standards were treated along with the samples for the color development. The absorbance was measured at 520 nm and a standard curve was plotted. The amount of sucrose was measured as fructose equivalents against the standard curve of fructose. Total fructose equivalents were converted into total sucrose by multiplying with the molar conversion factor, 1.9 for sucrose from fructose.

Starch estimation

Starch from the defatted meal of each sample was analyzed using the enzymatic procedure of Karkalas (1985). A sample of 125 mg finely ground defatted peanut meal was placed into the test tubes and 50 μL of α-amylase (Sigma Chemical Company, St. Louis, MO) and 8 mL of water was added and boiled for 10 min. The tubes were cooled and the

contents were transferred into 100 mL volumetric flasks and the volume was adjusted with distilled water. An enzyme blank was prepared by transferring 50 μL of α-amylase into a 100 mL volumetric flask and the volume was made up with water. Both the samples and the enzyme blank were filtered into conical flasks and in duplicate, 1 mL of each filtrate was transferred into appropriately labeled test tubes. To each test tube 1 mL of amyloglucosidase in citric acid buffer (pH 4.6) was added and the tubes were heated at 60°C for 30 min. Tubes were cooled to room temperature and to each tube 8 mL of water was added and vortexed. From each sample and enzyme blank, 1 mL of hydrolysate was transferred into a new test tube and to each 5 mL of glucose-oxidase-peroxidase chromogen (Karkalas, 1985) was added. Tubes were immersed in the water bath at 35 °C for 40 min in dark until a pink color developed. After cooling to the room temperature the absorbance was measured at 505 nm.

The starch content was calculated as g/100g of defatted sample with the formula, (As - Ae) X 9000/Ag X mg sample, where As and Ae are the absorbance of sample and enzyme blank respectively and Ag is the absorbance of standard solution containing glucose 100 μg mL^{-1}.

Ash and moisture content determination

Moisture and ash concentrations were determined following official moisture procedure (27.005) and ash

determination procedure (27.009) respectively (AOAC, 1984). Mineral (ash) was determined on the defatted meal.

Statistical analysis

Thirteen shriveled-seed classes from three shriveled-seeded lines comprising five classes from 529B, five classes from 563A and three classes from 647A and one normal-seeded class from Sunrunner were analyzed for individual chemical components separately, using a randomized complete block design with four replications. The analysis of variance was determined using SAS General Linear Models program (SAS Institute Inc., Cary, NC). Multiple comparisons were made using Duncan's multiple range test using the SAS GLM procedure. Means for total protein, sucrose, starch and ash were reported on dry weight basis on the defatted meal.

Experiment 2

Plants from both 529B and Sunrunner were harvested at 140 DAP from a larger experiment planted on May 5, 1993 at the University of Florida Green Acres Agronomy Farm, Gainesville. Collection of mature seed and classification of mature seed of the shriveled-seed line, 529B into five shriveled-seed classes was done exactly as described for Experiment 1. The seed was stored at -20°C until used for lipid and protein profile analysis.

Estimation of triacylglycerols and phospholipids

Lipid from each of the five shriveled-seed phenotypic classes of line 529B and from the mature seed of Sunrunner was extracted by following the same procedure described in the Experiment 1. Lipid was stored at -20 °C until used.

Approximately 50 mg of lipid in 100 uL of hexane was streaked on precleaned 20 X 20 silica-coated TLC plates (Fisher Scientific) using a streaker. Each sample was streaked in duplicate with the same amount of lipid and developed under similar conditions. The plates were developed in tanks saturated with hexane : diethylether : acetic acid (80 : 20 : 1 v/v) and sprayed with 0.1% dichlorofluorescein in ethanol followed by drying for a brief period. The triacylglycerol and phospholipid bands were detected under ultraviolet light and the two bands were marked and scraped into two separate test tubes. The lipids were extracted twice using chloroform : methanol (2:1). The samples were evaporated to dryness under N_2 stream and fatty acid methyl esters were prepared from each lipid fraction using the method by Maxwell and Marmer (1983). Each sample was dissolved in 1 mL of isooctane containing tricosanoic acid methyl ester internal standard at the concentration of 2.004 mg/mL, and to this 100 μL of 2N KOH in methanol was added and the tubes were vortexed for 60 sec then centrifuged. The lower methanol layer was discarded and 0.5 mL of saturated ammonium acetate was added, vortexed and

then centrifuged and the lower aqueous layer was discarded. The sample was washed one more time with water, centrifuged and the lower water layer was discarded. A small amount of sodium sulfate was added, the tubes were centrifuged and the top layer was removed and 2 μL sample was injected into GC equipped with FID and split injector. The column used was a DB-225 30 m X 0.32 mm, i.d 0.25 μm film (J&W, Rancho Corva, CA) operated with helium carrier gas at a linear flow velocity of 22 cm/sec. The column was programmed from 195 C to 225 C at 2 C/min. Total weight of fatty acids in each lipid class was calculated by comparing the total peak area of the sample with the peak area and the amount of internal standard used in each sample. Total weight of phospholipids were reported as dioctadecanoic acid phophatidalethanolamine, using a conversion factor of 1.2939.

Protein extraction and electrophoresis

Approximately 2 g of seed from each shriveled-seed class was ground to a fine powder in liquid N_2 and the powder was mixed into homogenizing buffer (pH 7.5) containing Hepes, EDTA and proteolytic inhibitors, PMSF, PVPP and DTT. The slurry was centrifuged at 25,000 g for 45 min at 4 °C. The supernatant was collected as aliquots into plastic culture tubes and stored at -20 °C. Protein was estimated using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Thirty μg of protein from each sample was diluted twice with the sodium dodecyl sulfate (SDS) reducing buffer consisting of 0.5 M Tris-HCl pH 6.8, 10% SDS, and ß-mercaptoethanol with 0.1% bromophenol blue tracking dye. The samples were mixed well and heated in a boiling water bath for 4 min, centrifuged for 1 min and loaded on 10% polyacrylamide discontinuous gel system of Laemmli (1970). The gels were stained for 1/2 hr in 0.1% Coomassie Blue R-250, 40% methanol and 10% acetic acid solution and destained for 3 hr in 40% methanol and 10% acetic acid to remove the background. The molecular weights of polypeptides were determined by calculating the Rf values of all major bands and by using the standard curve plotted with Rf values against log of molecular weight for each of the standard protein markers (Bio-Rad Laboratories, Hercules, CA).

Experiment 3

Developmental studies

Plants were harvested at 97 DAP from the shriveled-seeded line 529B planted on May 5, 1993 at University of Florida Agronomy Farm, Gainesville. The seeds were immediately hand-shelled and grouped into four maturity groups according to their maturity level based on the inner shell darkness and testa color (Table 4-1). Each group was thoroughly examined for shriveling characteristics by dissecting the seed horizontally into two halves. Shriveled cotyledons with thin, concave inside with brittle texture

Table 4-1. Classification of peanut seed developmental stages based upon inner pericarp and testa characteristics.

Stage	Inner Pericarp Characteristics	Testa Color
Stage I	White, fleshy	White, thick
Stage II	White, beginning to dry, cracks are seen	White but pink at the end
Stage III	White, dry, turning light brown	Light pink
Stage IV	Dry, brown all over	Dark pink

were grouped as shriveled types. Normal cotyledons were less concave inside and more fleshy. The classification into intermediate and mature stages was easier because the shriveling of the cotyledons was very conspicuous even though the seed were fresh.

Two classes of cotyledons from each maturity group were collected and the seed coats were removed and stored at -70 °C until used.

Estimation of triacylglycerols and phospholipids

Estimation of triacylglycerols and phospholipids for each class at each maturity stage was performed by following the method described in the Experiment 2.

Protein extraction and electrophoresis

Protein extraction, quantification and electrophoresis were done according to the method described for Experiment 2, except for the amount of protein loaded on the gels. Only 20 μg of protein from each sample was loaded on each track of the gel.

Results and Discussion

Experiment 1

Pooled data of all thirteen shriveled-seed classes from the three shriveled lines and from Sunrunner were analyzed for each seed component. The only alternative method of statistical analysis of these data would be to use unbalanced split plot technique with missing data. Because

the shriveled-seeded line 647A did not possess the first two phenotypic seed classes, the lack of expression of these two classes should not be considered as missing data for statistical analysis. Therefore, the thirteen shriveled-seed classes and one normal class from Sunrunner were analyzed with each as an independent treatment in a randomized complete block design. Mean comparisons among these treatments were performed using Duncan's multiple range test. This test can also provide information about whether or not a particular phenotypic seed class in each one of the shriveled lines falls into the same Duncan's group.

The total protein, sucrose, starch, mineral were determined and reported on the defatted meal basis because, there were no significant differences observed in the concentration of starch and mineral. Therefore, the differences in the concentration of protein and sucrose may reflect the changes in the concentration of these components in each seed class, even on a defatted meal basis. These results can also be reported on whole meal basis, by multiplying the concentration of these components with a fraction, (1-lipid concentration) for the respective seed class.

Analysis of variance for total moisture concentration

Moisture concentration was determined on each phenotypic class of seed that had been maintained at room

temperature after they were shelled from the pods dried under uniform drying conditions. Analysis of variance showed that there were significant differences in the moisture level among the different shriveled-seed classes (Table 4-2). Overall, moisture concentration in the Class 1 was higher than Classes 4 and 5 (Table 4-3). Figure 4-3 shows the Duncan grouping of each class of shriveled seed for its moisture concentration.

Analysis of variance for total oil

Significant differences were also found for the total oil concentration among the phenotypic seed classes (Table 4-2). In all of the shriveled lines, a lower mean oil percentage was observed in Class 1 seed compared to Class 5 seed. Oil concentrations in Class 2, Class 3 and Class 4 were intermediate between Class 1 and Class 5 (Table 4-3). Figure (4-4) shows the Duncan grouping for each class of shriveled seed, based upon the lipid concentration.

Analysis of variance for total protein

Total protein concentration in the defatted meal was also significantly different among the seed classes (Table 4-2), with a lesser concentration of protein in Class 1 seed (Table 4-3). Figure (4-5) shows the Duncan grouping of shriveled-seed phenotypic classes for the total protein concentration.

Table 4-2. Analyses of variance for total lipid, protein, sucrose, starch, mineral and moisture concentration of the fourteen shriveled-seed phenotypic classes from three shriveled-seeded lines and Sunrunner.

Source of Variation	Degrees of Freedom	Mean Squares					
		Lipid	Protein	Sucrose	Starch	Mineral	Moisture
Block	3	3.94	7.10	13.17	0.86	1.57	5.01
Phenotypic shriveled seed class	13	252.14**	128.38**	122.86**	1.89NS	1.19NS	16.96**
Error	39	2.72	1.71	4.98	0.99	0.15	2.55

**, NS indicate significance and non-significance of F-test at 0.01% level

Table 4-3. Total lipid, protein, mineral, moisture, sucrose, and starch in fourteen shriveled-seed phenotypic classes from three shriveled seeded lines and Sunrunner. Only lipid is on a dry weight basis. Other components are on defatted meal basis and could be expressed per unit dry weight by multiplying by (1-lipid fraction) from each class.

Phenotypic Shriveled Seed Class		Concentration					
		Lipid		Protein*		Mineral*	
		Mean	SE	Mean	SE	Mean	SE
		g 100 g^{-1}					
1.	529B-1	28.92	0.57	38.41	0.48	4.50	0.01
2.	2	36.56	0.60	43.77	0.31	4.78	0.23
3.	3	40.53	0.47	45.29	0.66	4.87	0.11
4.	4	45.20	1.26	51.09	0.78	4.86	0.06
5.	5	48.64	1.46	51.63	0.50	4.96	0.11
6.	563A-1	28.19	1.03	36.93	0.55	4.36	0.02
7.	2	37.12	0.28	39.72	1.18	4.54	0.13
8.	3	36.31	0.64	41.16	0.48	4.39	0.06
9.	4	45.07	0.79	47.72	0.69	4.51	0.14
10.	5	52.06	0.37	51.69	0.73	4.40	0.44
11.	647A-3	40.77	0.41	45.09	0.73	4.64	0.43
12.	4	47.66	1.44	50.83	1.10	4.91	0.05
13.	5	51.09	0.62	51.60	0.37	5.09	0.03
14.	Sunrunner	51.42	0.51	53.89	0.95	4.96	0.14

SE = Standard error of variance
* Defatted meal basis

Table 4-3 -- Continued.

Phenotypic Shriveled Seed Class		Concentration					
		Moisture		Sucrose*		Starch*	
		Mean	SE	Mean	SE	Mean	SE
		g 100 g^{-1}					
1.	529B-1	16.07	0.87	20.29	1.12	6.48	0.49
2.	2	12.64	0.20	16.95	1.34	6.49	0.62
3.	3	13.76	1.00	16.31	1.58	7.15	0.53
4.	4	12.45	0.60	10.25	1.36	6.75	0.50
5.	5	11.24	1.07	8.52	0.88	6.85	0.60
6.	563A-1	18.36	1.43	23.82	1.89	5.98	0.27
7.	2	14.05	0.59	22.24	1.21	5.92	0.19
8.	3	14.29	1.18	18.34	1.36	6.67	0.22
9.	4	12.91	0.52	14.85	0.63	6.80	0.29
10.	5	11.44	0.99	8.46	0.55	7.44	0.23
11.	647A-3	14.27	0.94	17.73	1.73	7.90	0.82
12.	4	13.24	0.40	11.15	0.67	7.85	0.67
13.	5	10.96	0.30	8.72	0.21	7.92	0.71
14.	Sunrunner	11.12	0.03	7.70	0.51	7.77	0.11

SE = Standard error of variance
* Defatted meal basis

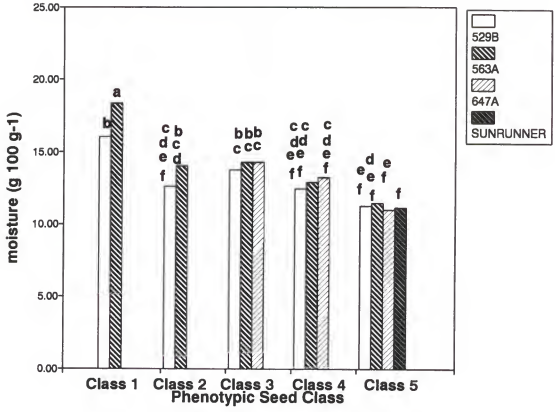

Figure 4-3. Duncan grouping of phenotypic shriveled-seed classes for moisture concentration.

Seed classes followed by the same letter are not significantly different at 0.01 probability.

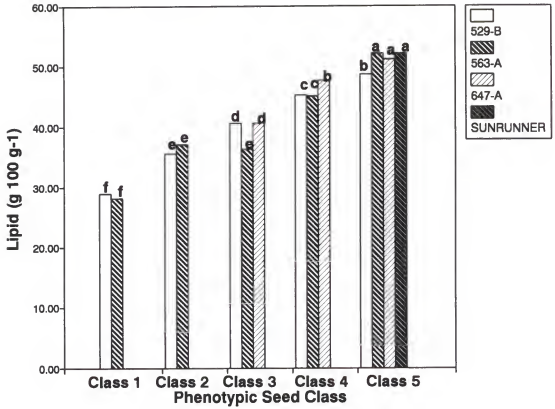

Figure 4-4. Duncan grouping of phenotypic shriveled-seed classes for lipid concentration.

Seed classes followed by the same letter are not significantly different at 0.01 probability.

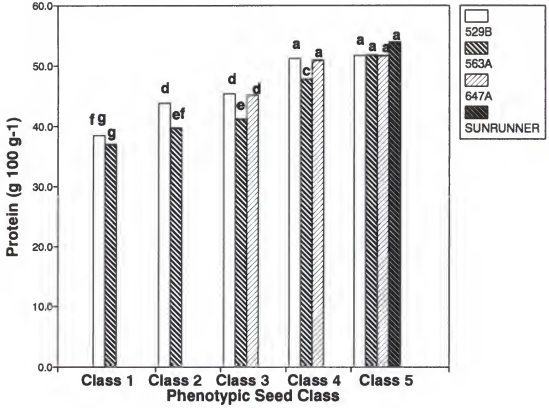

Figure 4-5. Duncan grouping of phenotypic shriveled-seed classes for total protein concentration.

Seed classes followed by the same letter are not significantly different at 0.01 probability.

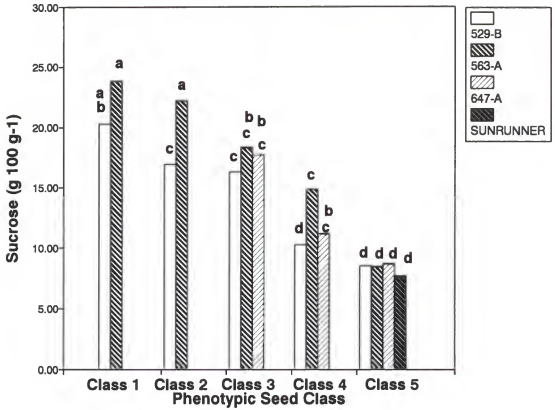

Figure 4-6. Duncan grouping of phenotypic shriveled-seed classes for sucrose concentration.

Seed classes followed by the same letter are not significantly different at 0.01 probability.

Analysis of variance for sucrose

Sucrose concentration in the defatted meal was significantly different among the shriveled-seed classes (Table 4-2). Class 1 seed showed higher levels of sucrose compared to the Class 4 or Class 5 seed (Table 4-3). Figure (4-6) shows the Duncan grouping of shriveled-seed classes for sucrose concentration.

Analysis of variance for starch

No significant differences for starch concentration was observed among the seed phenotypes (Table 4-2) when reported on the defatted meal basison defatted meal basis (Table 4-2).

Analysis of variance for mineral concentration

No significant differences were observed for the mineral concentration among the shriveled-seed classes (Table 4-2) as observed on defatted meal basis.

The overall data in the seed composition study indicate that the shriveled-seed phenotypes contained a greatly reduced level of lipid concentration and almost three times higher concentration of sucrose as compared to their normal counterparts. The moisture concentration in the most shriveled-seed classes was significantly higher than normal-seed classes. This increased hygroscopicity of these kernels may be due to the presence of higher concentration of sucrose and lower amount of oil in these seed. While comparing the sound and shriveled kernels of peanut obtained

from commercial lots, Chiou et al. (1992) also showed that shriveled kernels with higher levels of sucrose and lower lipid concentration had a higher moisture content than sound kernels.

In the case of wrinkled peas (Kooistra, 1962), and shrunken mutants sh2, bt1 and bt2 mutants in maize (Creech, 1965), the increased concentration of sucrose was due to a metabolic lesion in the starch production. In the ra locus of wrinkled peas, deficiency of a starch-branching enzyme caused a lower rate of branching and thereby provided fewer ends of glucose chains as substrates for starch synthase (Bhattacharyya et al., 1993). This affected the overall starch synthesis and led to an increase in sucrose levels (Edwards et al., 1988). In the rb locus the increased levels of sucrose and decreased starch concentration are due to a reduced activity of the enzyme ADP-pyrophosphorylase (Smith et al., 1989). Similarly, the loss of activity of sucrose synthase in the sh-1 mutant (Chourey and Nelson, 1976), and ADP-glucose pyrophosphorylase activity at sh-2 and bt-2 mutants (Hannah and Nelson, 1976) results in the reduction of starch synthesis and accumulation of sucrose in the maize endosperm. While starch is the main storage product in both maize and pea, oil is the main storage product in peanut, constituting about 50% of total dry weight of the kernel. As sucrose is the primary carbon source for both starch and lipid biosynthesis, a lesion in

the metabolic pathway of any one of these two products can cause accumulation of sucrose in the seed. The shriveled-seed phenotypic classes had similar starch concentration. Therefore the increased sucrose concentration in the shriveled seed could possibly be due to a lesion in the metabolic pathway of conversion of sucrose into lipid. As there were no observable differences in the fatty acid composition of shriveled phenotypes compared to the normal phenotype (next section), this lesion could possibly be in the biosynthesis and rate of accumulation of the storage lipid.

The total protein concentration was significantly different among the shriveled-seed phenotypes ($P=0.0001$). The protein concentration was lower in the most shriveled classes than in the normal types. This situation is comparable with the other shriveled-seed mutants of pea and maize. The mean legumin content of round seeded lines of pea was about twice that of wrinkled seeded lines (Davis, 1980). Shrunken kernel mutants of maize are also characterized by lower levels of the storage protein, zein (Lee and Tsai, 1984). Turner and co-workers (1990) found that the difference in the levels of legumin in the wrinkled vs. normal pea lines was not due to the difference in the rate of mRNA synthesis but due to rapid mRNA degradation in the wrinkled seed types. Their *in vitro* studies with high concentrations of sucrose showed a decrease in legumin

transcripts, indicating that the transcripts are selectively unstable at more negative osmotic potential. A similar finding was obtained from the studies of Rocha-Sosa et al. (1989) in the case of potato storage protein at higher sucrose levels. As both legumin and zein proteins are the major storage proteins in pea and maize respectively, any decrease in the levels of these protein is reflected in the total protein concentration of the seed. If this were the case with the shriveled-seed mutants of peanut, the higher sucrose levels in these mutant seed might have been affecting the accumulation of major seed storage proteins of these seed.

Experiment 2

The relative weight of phospholipids to the amount of triacylglycerols in each shriveled-seed phenotypic class is presented in Table 4-4. Each value is a mean of two independent observations from two TLC replications. The phospholipid concentration was the highest in the most shriveled class and decreased as the seed phenotype became more normal. The phospholipid concentration in Class 5 was comparable with that of Sunrunner seed and agreed well with the phospholipid values reported by Sanders (1980) for fully mature peanut seed.

A similar type of increase in phospholipid concentration was found in wrinkled pea (*Pisum sativum* L.)

(Coxon and Davis, 1982), shrunken kernel mutants of maize (Flora and Wiley, 1972), and barley mutant, Risφ 1508 (Bhatty and Rossnagel, 1980). However, in all these mutations the storage lipid content was increased as well. In wrinkled peas, although the mechanism by which the storage lipid content increased is not known, it is likely that a greater membrane area due to increased cell volume because of higher water content can result in higher levels of phospholipids (Bettey and Smith, 1990).

The fatty acid composition of total lipid (Table 4-5) from each shriveled-seed class was not different from that of triacylglycerol fraction (Table 4-6) from each class. This is to be expected given the fact that triacylglycerol predominates the peanut seed lipid. Also the shriveled classes were not different among themselves for fatty acid composition.

The lower total lipid concentration and increased levels of phospholipid, associated with no observable changes in the fatty acid composition of the shriveled seed, implies that the storage lipid synthesis is severely affected at one of the key regulatory steps leading to storage lipid biosynthetic pathway rather than at fatty acid synthase system *per se*. Several studies implicate acetyl-CoA carboxylase as a point of regulation of fatty acid synthase system (see Ohlrogge et al., 1993; Slabas and Fawcett, 1992). The regulation of the rates of

triacylglycerol biosynthesis in oil-storing tissues has been suggested to be under the control of the step catalyzed by the enzyme phosphatidate phosphatase (see Frentzen, 1993). In developing seeds of groundnut (*Arachis hypogaea* L.), the activity of the microsomal phosphatidate increased with the active triacylglycerol deposition (Sukumar and Sastry, 1987).

Besides the tight regulation of rate of fatty acid biosynthesis *per se* or triacylglycerol biosynthesis on the whole, the rate of synthesis and availability of acetyl-CoA may have important consideration in the utilization of carbon source for seed lipid biosynthesis. Contrary to the view expressed by Slabas and Fawcett (1992) and Browse and Somerville (1991) about the translocation of free acetate into the plastid for fatty acid synthesis in the seed tissue, Ohlrogge et al. (1993) in their recent review presented the results of several studies indicating the plastids of oilseeds to have a complete glycolytic pathway. This together with pyruvate dehydrogenase can convert the glucose to acetyl-CoA within the plastid for fatty acid synthesis. Alternatively, sucrose imported into the cytoplasm could be converted into malate with cytoplasmic glycolysis. Malate then enters the plastid where malic enzyme converts it to pyruvate followed by production of acetyl-CoA by pyruvate dehydrogenase. If there is any block in one of these several alternative metabolic pathways that

Table 4-4. Relative weight percentage of total phospholipid to total triacylglycerol in five shriveled-seed phenotypic classes of line 529B and Sunrunner.

Seed Phenotype	Relative Weight % (Phospholipid/ Triacylglycerol) X 100
	%
Class 1	3.34
Class 2	1.45
Class 3	1.44
Class 4	0.74
Class 5	0.51
Sunrunner	0.66

Table 4-5. Fatty acid composition of total lipid from five shriveled-seed phenotypic classes of line 529B and Sunrunner.

Seed Class	Fatty acid							
	16:0	18:0	18:1	18:2	20:0	20:1	22:0	24:0
	Wt %							
Class 1	11.4	3.3	47.9	28.0	1.4	1.6	2.7	2.2
Class 2	10.5	3.6	49.6	27.7	1.6	1.3	2.8	2.0
Class 3	8.5	3.7	63.9	15.9	1.6	1.3	2.8	1.8
Class 4	8.9	3.3	56.7	23.9	1.5	1.1	2.8	1.4
Class 5	10.8	2.8	46.1	33.2	1.4	1.1	2.6	1.3
Sunrunner	8.8	2.6	59.1	21.8	1.3	1.3	2.6	1.6

Table 4-6. Fatty acid composition of triacylglycerol from five shriveled-seed phenotypic classes of line 529B and Sunrunner.

Seed Class	Fatty acid							
	16:0	18:0	18:1	18:2	20:0	20:1	22:0	24:0
	Wt %							
Class 1	10.1	3.4	47.4	27.5	1.7	1.8	3.4	2.9
Class 2	8.5	3.7	56.3	21.3	1.8	1.5	3.3	2.3
Class 3	8.6	3.7	56.4	21.3	1.7	1.5	3.3	2.3
Class 4	8.0	3.4	56.7	23.3	1.7	1.2	2.9	1.7
Class 5	9.6	2.9	45.8	32.9	1.6	1.2	3.2	1.8
Sunrunner	8.2	2.6	58.8	21.4	1.5	1.4	3.0	1.9

operate in peanut seed lipid biosynthesis, the level of sucrose into the seed tissue may increase. Alternately, the acetyl-CoA pool, that is distinctly maintained for fatty acid synthesis (Ohlrogge et al., 1993), may act as a feedback regulator when it cannot be incorporated into the fatty acid biosynthesis, which in turn might result in the accumulation of sucrose in the seed tissue. As most of the shriveled seed on a shriveled seed plant are produced at the latter part of the reproductive stage (Chapter 2), the reduction in the storage lipid synthesis and accumulation of sucrose in the shriveled seed might be under the control of basic developmental regulatory mechanism of the plant.

The protein profiles of five shriveled-seed classes from the shriveled-seeded line, 529B showed differences in the quantity of major proteins at approximate molecular weights 47.2 kd, 43.2 kd, 37.5 kd and 20.5 kd and a slight variation in 25.8 kd protein, designated as b, c, d, e, and f respectively, in Figure 4-7. No remarkable differences were observed in the quantity of major protein, 'a' of approximately 74.9 kd molecular weight among the five shriveled classes. However, the studies of Chiou et al. (1992) on shriveled peanut kernels from commercial lots found no visible differences in quantitative or qualitative characteristics of proteins.

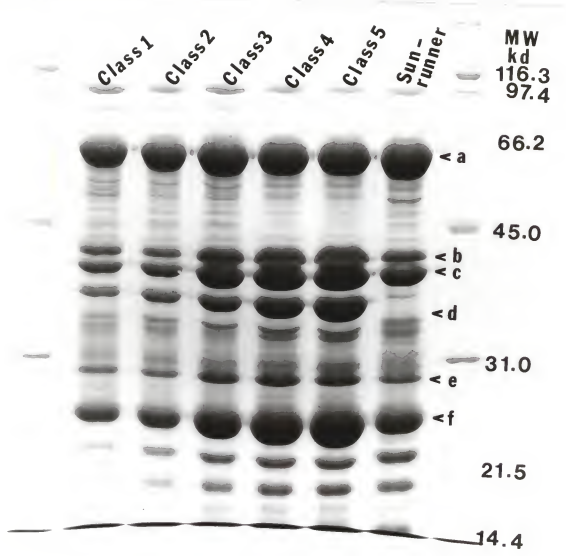

Figure 4-7. SDS-PAGE protein profiles of five shriveled seed phenotypic classes of 529-B and Sunrunner. Left and right lanes contain the molecular markers.

Proteins a, b, c, d, e, f represent 74.9 kd, 47.3, 43.2 kd, 37.5 kd, 25.9 kd and 20.5 kd approximate molecular weight proteins respectively.

Based on the approximate molecular weights (McWatters and Cherry, 1982; Yamada et al., 1979; Krishna and Mitra, 1987; Krishna et al., 1986), and gel banding patterns (Krishrna and Mitra, 1987; Krishna et al., 1986) protein 'a' was tentatively identified as α-conarachin subunit and proteins b, c, and d were acidic, and protein 'f' was basic subunit of storage protein arachin. The reduced levels of proteins b, c, d, e, f and other minor proteins (Fig. 4-3) in the most shriveled phenotypic classes might be associated with the lower total protein concentration of these seed (Experiment I). Also, the protein 'd' (molecular weight 37.4 kd) is missing in the gel patterns of normal-seeded Sunrunner. Krishna et al. (1986) showed there is genetic variation for the presence or absence of acidic subunits of arachin protein in peanut.

The storage globulins, arachin and conarachin of peanut are often compared to legumin and vicilin proteins in other legume species. Studies of Turner et al. (1990) showed that there was a marked decrease in legumin protein accumulation in the wrinkled-seeded pea genotype (rr) compared to the round-seeded (RR) genotype. The accumulation of vicilin did not differ greatly between the two genotypes. The run-on transcription analysis of near isolines of R-locus shown very similar rates of transcription of legumin genes and suggested that the differences in legumin mRNA levels between the near isolines result from increased rate of

degradation of legumin mRNA in rr genotype. The storage protein mRNA levels were shown to be decreased in the round-seeded embryos cultured at higher sucrose culture medium, but the effect was more on legumin mRNA than vicilin mRNA levels. However, the mechanism by which sucrose differently affects storage protein mRNA levels is not known. The increased sucrose levels and decreased levels of tentatively identified subunits of shriveled-seed arachin protein patterns on SDS-PAGE may resemble the accumulation pattern of legumin in the rr genotype of pea (*Pisum sativum* L.). Similarly, the major protein 'a', identified as a-conarachin was relatively unaffected by higher sucrose concentrations, as in the case of vicilin gene product in pea.

Recent molecular studies of Paik Ro (1992) with seed cDNA clones showed the accumulation pattern of arachin protein to reveal a sequence similarity of vicilin gene. If the nucleotide sequence of arachin gene resembles that of vicilin, then it will be interesting to know the differential responses of the gene products in different legume species.

Experiment 3

Developmental studies

The shriveled and normal-seed samples obtained at four developmental stages, based on fresh seed morphology were used to investigate the differences in the changes in lipid and protein profiles during the course of seed development.

Except for the third developmental stage, in all other developmental stages, the relative weight of phospholipid to triacylglycerol was always higher in shriveled seed than in normal seed (Table 4-7). This trend may reflect that the shriveled phenotypes are not capable of accumulating triacylglycerols from the very beginning of seed development. The fatty acid composition of the triacylglycerol fraction of both shriveled and normal seed were very similar at any particular stage. As has been observed before (Sanders et al., 1982) the palmitic acid and linoleic acid decreased slightly and oleic acid increased as the seed matured (Table 4-8).

A very similar trends to that of storage lipid accumulation has been observed with the accumulation of various polypeptides in the protein at various developmental stages of the shriveled phenotypes (Fig. 4-4). At any particular stage of development, the amount of proteins, b (45.2 kd), c (42.3 kd), d (36.2 kd), e (25.4 kd) and f (20.4 kd) were lower in the shriveled classes compared to the normal-seed proteins but there was no difference in the apparent amount of protein `a' (75.3 kd). However, the pattern of various polypeptide accumulation over developmental stages in the shriveled seed compared to the normal seed suggests that no protein is missing in the shriveled seed, but only the relative quantity of particular proteins is reduced.

Table 4-7. Relative weight percentage of total phospholipid to total triacylglycerol in shriveled and normal seed of line 529B at various stages of seed development.

Seed Developmental Stage	Relative Weight % (Phospholipid/ Triacylglycerol) X 100
	%
Stage I	
Shriveled	7.21
Normal	6.18
Stage II	
Shriveled	4.33
Normal	2.85
Stage III	
Shriveled	0.89
Normal	1.98
Stage IV	
Shriveled	2.28
Normal	0.75

Table 4-8. Fatty acid composition of triacylglycerol from shriveled and normal seed from line 529B at various stages of seed development.

	Seed Developmental Stage							
	I		II		III		IV	
Fatty acid	Shri-veled	Nor-mal	Shri-veled	Nor-mal	Shri-veled	Nor-mal	Shri-veled	Nor-mal
	Wt %							
16:0	14.1	15.1	12.7	12.4	9.6	9.3	9.6	10.3
18:0	2.1	2.1	2.8	2.1	2.9	3.1	2.7	3.5
18:1	34.7	31.9	41.0	39.1	48.0	54.8	46.0	45.3
18:2	35.9	36.1	30.1	33.6	29.9	23.2	33.4	29.2
20:0	1.4	1.4	1.7	1.5	1.7	1.7	1.5	1.8
20:1	2.0	2.1	1.8	1.8	1.3	1.4	1.2	1.6
22:0	6.8	7.6	5.1	5.8	3.5	3.6	3.0	4.0
24:0	2.6	2.6	2.8	2.6	2.0	2.2	1.7	2.8

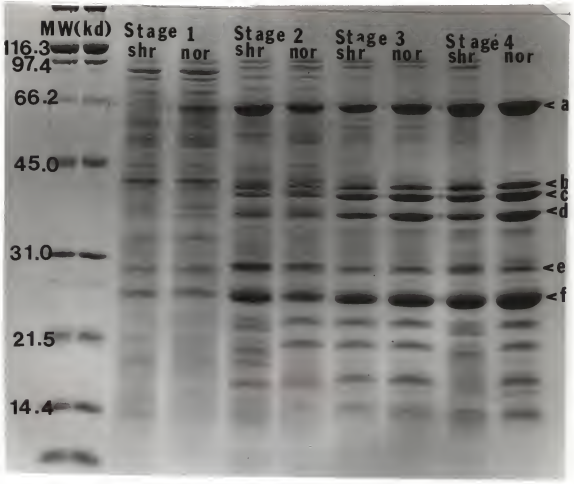

Figure 4-7. SDS-PAGE protein profiles of stage 1, stage 2, stage 3 and stage 4 developmental stages in shriveled (shr) and normal (nor) seed from 529-B. Left lane contains the molecular weight markers.

Proteins a, b, c, d, e, f represent 75.3 kd, 45.2, 42.4 kd, 36.3 kd, 25.5 kd and 20.4 kd approximate molecular weight proteins respectively.

Obviously more studies are needed to understand controlling mechanism regulating the accumulation of seed storage lipid and protein in the shriveled seed. Comparison of activities of various enzymes involved in lipid biosynthetic pathway in shriveled seed vs. normal seed from the same genotype might provide improved understanding about the lesion in the lipid biosynthesis in the shriveled seed.

CHAPTER 5
GROWTH AND PARTITIONING CHARACTERISTICS OF SHRIVELED SEED GENOTYPES

Introduction

Three breeding lines, 529B, 563A and 647A in the Florida peanut breeding program show characteristic seed shriveling even after full maturity. These lines apparently produce a large number of pods and more vegetative growth than most normal-seeded lines. All of these genotypes are characterized by various levels of seed shriveling associated with lower levels of oil and storage protein and increased accumulation of sucrose in the seed (Chapter 4). To identify the physiological mechanism responsible for the distinct vegetative and reproductive characteristics of these genotypes, a growth and partitioning analysis was conducted using shriveled line 529B and normal-seeded cultivar, Sunrunner.

Differences in the reproductive growth rates and the partitioning of photosynthetic assimilate to the pods and seeds between normal and shriveled-seed genotypes may indicate whether the seed shriveling is caused by a source related defect or a general metabolic lesion in the

utilization of the photosynthate for the normal growth of the seed.

Even though no specific studies exist for the genotypic differences for seed growth *per se*, differences have been observed for both vegetative and pod growth characteristics in peanut. Duncan et al. (1978) showed that peanut lines which differ by pod yield potential also differ in vegetative and reproductive (pod) characteristics.

Periodic increments in plant dry matter can be expressed as per unit leaf area or land area basis, thereby allowing comparisons of relative production efficiencies of different crops or of any given crop during varying growth stages (Pixley, 1985). Crop growth curves plotted with this data over times are characteristically sigmoid. There is a period of slow, logarithmic growth during which leaves develop and begin photosynthesis, or in case of seed, the lag phase includes cell division, cell number determination and cell expansion processes. Total crop growth becomes linear when a full leaf canopy is established. An apparent deviation from this maximum constant growth rate occurs after reproductive growth is initiated and the rate of total biomass production begins to decline. Linear pod growth occurs after the maximum pod number is established and pod filling begins.

Crop growth rate (CGR), vegetative growth rate (VGR) and pod growth rate (PGR) can be calculated by linear

regression of total, vegetative and pod biomass accumulation curves when they are in their respective linear phases (Pixley et al., 1989). Partitioning of photosynthate to pod growth can be estimated from the computed values of CGR and PGR, by dividing the PGR by CGR (Duncan et al., 1978). Duncan et al. (1978) and Knauft and Gorbet (1990) estimated partitioning of the total dry matter to the pods for several cultivars and breeding lines and showed that there are significant differences among various peanut genotypes for partitioning of photosynthetic assimilate to the pods.

Partitioning to reproductive growth can be computed using energy requirements for reproductive and vegetative tissues in terms of glucose equivalents. Penning De Vries et al. (1982) calculated the energy cost of producing different plant tissues. By using these glucose energy cost values of 2.556 g glucose/ g product for protein, 3.106 for lipid, 2.174 for lignin, 0.929 for organic acid and 0.05 for mineral, Boote et al. (1985) computed the synthesis cost for various peanut tissues. Using the estimated cost of seed as 2.54 g glucose g^{-1} of seed and 1.44 g glucose g^{-1} for the shell, Pixley et al. (1989) calculated assimilate partitioning to pod growth for four cultivars differing in leafspot resistance.

Calculating partitioning based on glucose cost equivalents is more correct than using dry matter values, especially when the vegetative and/or reproductive tissue

composition of the genotypes is different. As the composition of shriveled seed is different from the normal-seeded type, growth and partitioning analysis in this study was done using the glucose energy cost equivalents for the synthesis of vegetative and pod tissues. Both unadjusted growth rates computed with vegetative and pod dry matter and glucose energy-adjusted growth rates are presented and comparisons are drawn.

Materials and Methods

The shriveled-seeded line 529B and Sunrunner were grown in 1992 and 1993. The 1992 experiment was planted on May 19, 1992 at the University of Florida Green Acres Agronomy Farm, Gainesville, FL. The soil type is an Arredondo fine sand (loamy, siliceous, hyperthermic Grossarenic Paleudult). Seed were planted at the rate of 75 seed for each row, in two row plots 6.1 m long, spaced 0.91 m apart. Each genotype was planted in 6 row-plots and replicated four times in a randomized complete block design. Standard cultural practices were followed throughout the growing season.

Sampling for growth analyses began at 100 DAP and went until 160 DAP. A 1.0-m sample row was harvested from each replication at each 10-day interval. Adequate care was taken to collect pods that were dropped from the plants at later sampling dates due to disease or over maturity.

Immediately after harvest, pods were separated from the plants and vegetative portions and pod portions were dried separately at 60°C. Vegetative and pod dry weights were recorded from each sample, and pods were shelled using a sample sheller. Seed weight was recorded and the shell weight was computed as the difference between pod weight and seed weight. Shelling percentage was computed as the percentage of seed weight to the total pod weight. Seed samples from each field replicate were then used for estimating the proportion of individual chemical components, lipid, protein, cellulose carbohydrate, and ash from each sample.

The second year growth analysis was planted on May 5, 1993. This experiment was also conducted at the University of Florida Green Acres Agronomy Farm and the same planting and other cultural practices were followed as the previous years experiment. The sampling was done, beginning at 40 DAP until 170 DAP. The samples were collected and divided into vegetative and pod portions as described above.

Estimation of Individual Chemical Components

Seed from 529B from each harvest was a mixture of variously shriveled-seed phenotypes. Therefore the entire seed sample from each harvest was ground into a homogeneous meal to get uniform sample of the genotype for estimating each individual chemical component. Seed from Sunrunner

were also ground into uniform meal for the chemical analyses.

From each sample, 3 g of meal sample was taken to estimate lipid concentration using hexane extraction. The sample was first extracted two times using 10 ml hexane and after the second extraction, the meal was once again ground into a fine powder and one more time extracted with another 10 ml hexane. The solvent was evaporated under a stream of nitrogen and weight of the lipid was gravimetrically determined. The meal was evaporated to dryness. This defatted meal was analyzed for total protein and ash (mineral) concentration from the defatted meal as described in Chapter 4. The protein and ash values were adjusted to the whole meal basis by dividing by the fraction, (1-lipid fraction). Values for lignin and organic acid were obtained from Boote et al. (1985). Total cellulose-and-carbohydrate was obtained by subtracting all other component fractions from 1.00.

Computation of Glucose Equivalents

Glucose equivalents for each seed component were using calculated the glucose cost values given by Penning De Vries et al. (1982). The values were 2.556 g of glucose to produce 1 g of protein, 3.106 g glucose for 1 g lipid, 2.174 g glucose for 1 g lignin, 0.929 g glucose for 1 g organic acid, and 0.05 g glucose for 1 g mineral. Glucose cost

equivalent for each component fraction was calculated by multiplying the fraction times the corresponding glucose cost value. The total glucose equivalents for 1 g of seed was used to calculate the total seed glucose equivalents per 1 m^2. As the mutant plant is normal in appearance and vigor, the glucose cost for the synthesis of vegetative and shell biomass per 1 m^2 area were calculated according to Boote et al. (1985) and Pixley et al. (1989), by using the factor 1.44 g of glucose per g of each vegetative tissue synthesized.

Estimation of Partitioning of Photosynthate to Pod and Seed Growth

Partitioning of photosynthate to pod, seed and shell growth was estimated using the computed growth rates of each tissue, both on dry matter basis and glucose cost equivalent basis. Crop growth rate (CGR), vegetative growth rate (VGR), pod growth rate (PGR), seed growth rate (SGR) and shell growth rate (SHGR) were computed by linear regression of total, vegetative, pod, seed and shell biomass accumulation curves using the data from each replicate in the year 1993. Partitioning to pod was calculated by dividing the PGR by CGR. Similarly partitioning to seed and shell were calculated by dividing the respective growth rates by the CGR. Partitioning using the glucose equivalent values was also calculated in a similar fashion, using the respective glucose equivalent growth rates.

Individual analyses of variance was performed for each genotype on CGR, VGR, PGR, SGR and SHGR of each replicate, following a randomized complete block design as previously described by Pixley et al. (1989). The partitioning factors computed for pod growth, seed growth and shell growth from individual replicate values for the respective growth rates were also subjected to similar type of statistical analyses as described by Pixley et al. (1989).

Results and Discussion

The vegetative, pod, seed and shell dry matter data obtained from seven sampling dates from 1992 and fourteen sampling dates from 1993 were converted into respective vegetative, pod, seed and shell glucose cost equivalents and the mean glucose cost equivalents per m^2 land area for each sampling period are presented in Tables 5-1, 5-2, 5-3, 5-4. The shelling percentage calculated for two genotypes at each harvest date for both years showed that 529B always had a lower shelling percentage than Sunrunner (Table 5-5).

Growth Analysis and Partitioning

Growth analyses was performed using crop growth rate, pod growth rate, and vegetative growth on dry weight basis and on glucose energy cost equivalent basis showed no significant differences between the shriveled-seeded and normal-seeded genotypes (Tables 5-6 and 5-7). However,

Table 5-1. Mean glucose energy cost equivalents for the production of various biomass components per 1 m^2 land area in year 1992.

Days After Planting	Total Biomass				Vegetative Biomass				Pod Biomass			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	529B		Sunrunner		529B		Sunrunner		529B		Sunrunner	
	g glucose m^{-2}											
100	1256.5	217.4	1236.1	229.6	870.6	131.5	866.7	180.8	386.0	92.5	369.3	65.6
110	1223.5	71.3	1579.2	137.4	810.5	34.4	1029.3	102.6	412.9	39.7	568.2	66.8
120	1481.0	60.1	1642.8	310.8	923.3	94.5	986.8	162.1	557.7	72.0	656.4	150.5
130	1628.2	214.6	1852.9	147.5	919.5	161.2	1081.7	72.5	708.7	99.2	771.2	139.7
140	1764.9	98.0	1636.1	237.4	974.3	68.3	836.6	146.2	790.6	57.5	799.5	151.4
150	1508.2	133.1	1578.6	215.3	851.3	95.9	782.0	110.3	656.8	48.9	796.6	153.9
160	1613.6	148.2	1785.3	213.3	831.2	77.2	869.7	115.8	782.3	76.6	915.6	44.9

Table 5-2. Mean glucose energy cost equivalents for the production of pod biomass components per 1 m^2 land area in year 1992.

Days After Planting	Seed Biomass				Shell Biomass			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	529B		Sunrunner		529B		Sunrunner	
	g glucose m^{-2}							
100	236.4	53.3	261.1	50.7	149.6	41.7	108.2	18.0
110	268.7	45.9	443.4	49.3	144.3	22.4	124.8	17.7
120	354.7	81.7	505.4	144.9	203.0	13.1	150.6	5.7
130	427.7	85.9	634.1	93.0	281.0	67.0	137.2	53.4
140	530.5	43.5	675.8	128.1	260.1	20.4	123.7	25.7
150	456.8	68.7	681.3	128.9	199.9	66.0	115.3	27.3
160	544.1	51.6	771.3	32.5	238.3	25.3	144.2	12.9

Table 5-3. Mean glucose energy cost equivalents for the production of various biomass components per 1 m^2 land area in year 1993.

Days After Planting	Total Biomass				Vegetative Biomass				Pod Biomass			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	529B		Sunrunner		529B		Sunrunner		529B		Sunrunner	
	g glucose m^{-2}											
40	129.9	118.7	165.2	136.4	129.9	78.9	165.2	76.6	0	-	0	-
50	154.3	96.7	179.0	131.5	154.3	66.7	179.1	72.0	0	-	0	-
60	328.8	101.3	283.8	48.9	304.6	99.5	276.1	65.4	24.2	21.9	7.7	44.5
70	573.3	133.1	608.1	72.0	457.1	108.8	504.6	66.7	116.2	49.6	103.5	58.9
80	648.5	137.4	750.2	164.5	478.6	157.2	533.8	47.9	169.9	62.1	216.4	77.2
90	815.9	161.2	1020.2	136.7	517.4	87.6	649.2	61.3	298.5	55.8	370.9	62.3
100	1395.8	68.3	1525.4	180.5	834.3	69.2	867.4	78.9	561.5	62.5	657.9	52.1
110	1400.5	100.1	1520.6	115.2	807.3	77.2	812.1	97.8	593.1	48.3	708.5	67.2
120	1831.1	48.9	1760.7	58.9	1046.6	69.4	914.8	99.4	784.5	47.6	845.8	69.2
130	1614.7	65.8	1699.1	220.6	925.0	111.2	888.9	55.9	689.7	41.8	810.1	33.5
140	2058.5	152.6	1914.7	181.9	1091.5	82.6	956.4	49.6	967.0	52.3	958.3	52.0
150	1667.3	77.4	1966.9	172.3	842.5	96.4	995.4	87.3	824.8	69.2	971.5	48.2
160	1619.4	92.1	1502.4	116.2	778.5	91.3	733.1	66.4	840.9	39.5	769.3	37.5
170	1249.8	58.9	1278.0	103.8	613.5	57.6	745.0	78.5	618.2	43.2	533.0	44.7

Table 5-4. Mean glucose energy cost equivalents for the production of pod biomass components per 1 m^2 land area in year 1993.

Days After Planting	Seed Biomass				Shell Biomass			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	529B		Sunrunner		529B		Sunrunner	
	g glucose m^{-2}							
40	0	-	0	-	0	-	0	-
50	0	-	0	-	0	-	0	-
60	10.4	3.5	3.1	0.5	13.8	2.1	4.6	0.7
70	61.8	5.1	63.5	33.2	54.4	3.9	39.9	11.9
80	92.3	7.9	145.2	50.6	77.5	10.7	71.1	7.8
90	188.7	33.6	270.3	32.7	109.2	11.6	100.6	8.6
100	379.1	31.2	509.8	44.2	182.3	17.9	148.1	19.6
110	359.7	28.7	554.6	17.9	233.6	8.9	153.8	27.9
120	542.5	69.5	684.2	21.9	241.9	12.9	161.6	38.2
130	490.7	71.5	674.4	27.9	198.5	21.6	135.7	16.7
140	569.5	55.9	788.6	33.2	397.7	18.9	169.6	11.1
150	482.5	49.6	826.5	53.7	342.4	19.7	145.0	28.1
160	572.0	84.2	654.9	27.6	268.6	21.5	144.3	36.1
170	433.8	62.7	436.0	19.9	184.3	13.5	97.0	21.0

Table 5-5. Shelling percentages of line 529B and Sunrunner at different sampling dates in the growing seasons, 1992 and 1993.

Days After Planting	Growing Season			
	1992		1993	
	529B	Sunrunner	529B	Sunrunner
	%			
70	-	-	43.82	50.32
80	-	-	44.85	57.03
90	-	-	53.45	62.74
100	43.82	61.54	56.98	67.85
110	56.58	69.92	51.80	69.57
120	54.96	68.04	59.66	72.56
130	51.42	75.85	62.14	75.69
140	59.20	77.81	48.35	75.16
150	61.74	79.06	48.05	77.14
160	61.27	77.27	57.46	77.42
170	-	-	59.31	73.27

Values calculated as percentage of seed weight to the total pod weight.

in 1992, the seed growth rate and shell growth rate both on dry weight basis and in terms of glucose energy equivalents were significantly different and also the shell growth rate differed in 1993 (Tables 5-6 and 5-7).

Although the data obtained for total crop growth rates were not statistically different between shriveled line and Sunrunner in the respective years, Sunrunner showed higher crop growth rate in 1992 and lower crop growth rate in 1993, compared to the shriveled line, both on dry weight basis and in glucose cost equivalents (Tables 5-8 and 5-9) (Figures 5-1 and 5-3). The pod growth rate on dry weight basis was lower in Sunrunner for 1993, however, in glucose cost equivalents, it was higher (Table 5-8). This was probably because of the higher glucose energy values required to synthesize the seed tissue of Sunrunner, especially the lipid fraction, which is costly in terms of glucose expenditure. In terms of glucose equivalents, the seed growth rates were lower in the shriveled-seeded type but the vegetative and shell growth rates were higher (Table 5-8, 5-9), (Fig. 5-2, 5-4).

Even though the vegetative growth rates computed for 1993 data were within the range of values reported in the literature for both dry weight and cost-adjusted values, the total crop growth rate and pod growth rates were lower than the values reported (Pixley et al., 1989).

Table 5-6. Analysis of variance for genotype effect on vegetative and reproductive growth rates on the basis of dry matter accumulation.

		1992		1993	
Source	Degrees of Freedom	Mean Square	P-Value	Mean Square	P-Value
CGR	1	0.004	0.94	16.791	0.21
PGR	1	0.806	0.32	1.248	0.51
SGR	1	2.868*	0.01	1.805	0.16
SHGR	1	6.426*	0.01	5.661*	0.05
VGR	1	-	-	8.904	0.10

CGR = crop growth rate; PGR = pod growth rate; SGR = seed growth rate; SHGR = shell growth rate; VGR = vegetative growth rate.
* F-test significantly different at 0.1 level.

Table 5-7. Analysis of variance for genotype effect on vegetative and reproductive growth rates on the basis of glucose cost equivalents.

		1992		1993	
Source	Degrees of Freedom	Mean Square	P-Value	Mean Square	P-Value
CGR	1	2.298	0.37	13.158	0.47
PGR	1	0.015	0.95	0.409	0.84
SGR	1	8.820*	0.05	16.618	0.11
SHGR	1	8.799*	0.07	11.786*	0.05
VGR	1	-	-	17.82	0.10

CGR = crop growth rate; PGR = pod growth rate; SGR = seed growth rate; SHGR = shell growth rate; VGR = vegetative growth rate.

Table 5-8. Growth and partitioning characteristics of shriveled-seeded line 529B and normal-seeded Sunrunner on the basis of dry matter accumulation.

Growth Characteristics	1992		1993	
	529B	Sunrunner	529B	Sunrunner
Growth Rate				
			$g\ m^{-2}\ day^{-1}$	
CGR	8.29§	8.30	12.94	10.05
PGR	6.10	5.46	6.14	5.35
SGR	3.61	4.81	3.36	4.31
SHGR	2.48	0.69	2.86	1.17
VGR	-	-	6.81	4.70
Partitioning ¶				
			%	
Pod	74.42§	66.44	47.53	53.64
Seed	44.51	58.74	21.94	43.61
Shell	29.91	8.18	21.96	11.61
Vegetative	-	-	52.47	46.34

CGR = crop growth rate; PGR = pod growth rate; SGR = seed growth rate; SHGR = shell growth rate; VGR = vegetative growth rate.
§ Data represent means of four replications
¶ Partitioning factors were calculated as percentages of (PGR/CGR), (SGR/CGR), (SHGR/CGR), and (VGR/CGR) respectively.

Table 5-9. Growth and partitioning characteristics of shriveled-seeded line 529B and normal-seeded Sunrunner on the basis of glucose cost equivalents.

Growth Characteristics	1992		1993	
	529B	Sunrunner	529B	Sunrunner
Growth Rate				
			g m^{-2} day^{-1}	
CGR	14.22§	15.43	21.76	19.19
PGR	11.13	11.05	11.98	12.43
SGR	7.47	9.57	7.84	10.73
SHGR	3.58	1.48	4.11	1.69
VGR	-	-	9.80	6.82
Partitioning ¶				
			%	
Pod	78.35§	72.34	55.03	65.19
Seed	53.38	62.59	36.25	56.34
Shell	24.99	11.60	18.80	8.70
Vegetative	-	-	44.96	35.25

CGR = crop growth rate; PGR = pod growth rate; SGR = seed growth rate; SHGR = shell growth rate; VGR = vegetative growth rate.
§ Data represent means of four replications
¶ Partitioning factors were calculated as percentages of (PGR/CGR), (SGR/CGR), (SHGR/CGR), and (VGR/CGR) respectively.

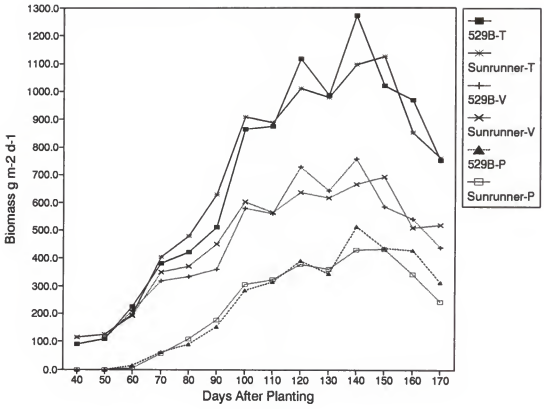

Figure 5-1. Accumulation pattern of total (T), plant (V), and pod (P) biomass in line 529B and Sunrunner at different intervals of crop growth in 1993.

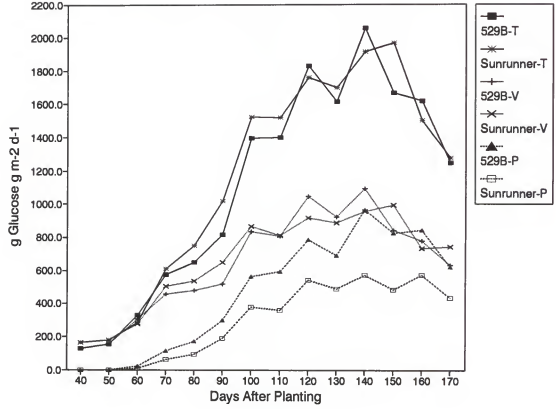

Figure 5-2. Accumulation pattern of total (T), plant (V), and pod (P) biomass in glucose equivalent costs of tissue produced in line 529B and Sunrunner at different intervals of crop growth in 1993.

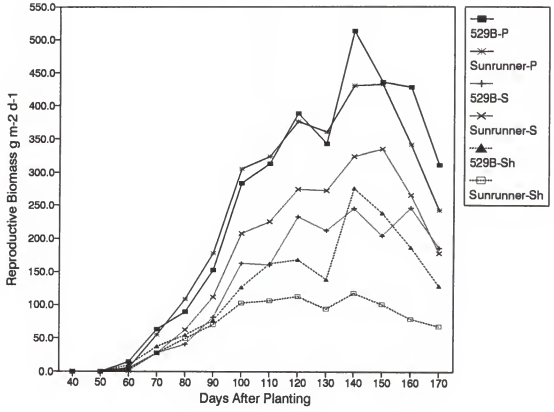

Figure 5-3. Accumulation of seed (S) and shell (Sh) biomass components in line 529B and Sunrunner in 1993.

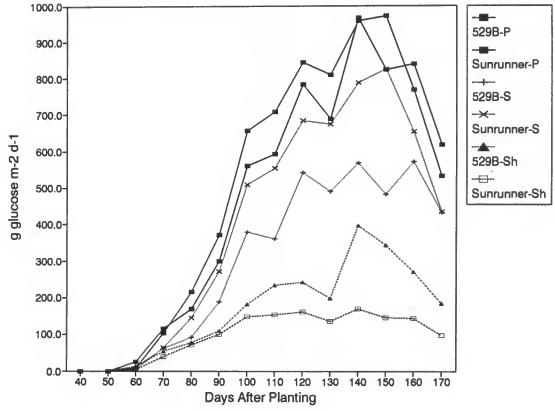

Figure 5-4. Accumulation of glucose cost equivalents into seed (S) and shell (Sh) biomass components in line 529B and Sunrunner in 1993.

This might be because of late sampling in the year 1992. The poor plant stand in 1993, might have caused more vegetative growth and less reproductive growth in both genotypes (Table 5-8 and 5-9).

The partitioning coefficients to pod and seed calculated using both dry weight values and glucose adjusted values were higher in the year 1992 (Table 5-8 and 5-9). The pod partitioning coefficient for Sunrunner on dry weight basis, in the year 1992 was comparable with the values reported by Knauft and Gorbet (1990). However, the partitioning coefficients for pod and seed were lower in the year 1993. This might be due to the poor plant stand in 1993, which allowed extended vegetative growth and thus lower partitioning of photosynthate to the pods in both genotypes.

The partitioning values for the pod and seed were higher for Sunrunner than for 529B on both dry weight basis and on adjusted values of glucose. The partitioning for shell and vegetative dry matter were higher in 529B (Table 5-8, 5-9).

These data indicate that the shriveled seed are not utilizing the assimilate supplied for seed growth, and thus have lower seed growth rates. Rather, the shriveled-seed genotypes produce a greater number of pods (and shells) spend the photosynthetic assimilate for shell and vegetative biomass production. Higher glucose equivalent values of

shell tissue suggests that there a block in the assimilate use for normal seed growth. There could also be a lesion in the assimilate transport process of phloem unloading; however, this is not consistent with high sucrose levels in the seed.

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BIOGRAPHICAL SKETCH

Lakshmi Rani Jakkula was born and brought up in Andhra Pradesh, India. During her childhood, several visits to her father's native village enriched her experience of rural Indian life. After finishing her SSC (10th Grade) in 1979, she chose biological sciences as her major for high school and graduated in 1981. She began her B.Sc. (Ag), in 1981 at S.V. Agricultural College, Andhra Pradesh Agricultural University, Tirupati, A.P. and graduated in 1985. As an undergraduate student, she was attracted to the elegance and beauty of the science of genetics, and began her M.Sc.(Ag) in genetics and plant breeding in 1985 and graduated in 1987. In the fall of 1990, she was accepted into the Agronomy Department at the University of Florida for her Ph.D. in plant breeding and genetics and started her research under the direction of Dr. David A. Knauft. She completed her Ph.D requirements in fall of 1994 and is interested in pursuing an academic career.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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